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DEGRADATION OF WHOLE CASEINS FROM RAW, PASTEURIZED AND HYDROGEN PEROXIDE TREATED MILKS BY CALF RENNIN AND A MICROBIAL COAGULANT

L. VÁMOS-VIGYÁZÓ, M. EL-HAWARY and E. KISS

(Received 29 August 1978; accepted 30 September 1978)

The actions of crystalline calf rennin (CR) and of a microbial milk clotting enzyme derived from *Endothia parasitica* (MR) on whole caseins prepared from raw, pasteurized (65 °C, 30 min) and hydrogen peroxide-treated skim milks (caseins Nos. I, II and III, resp.) were compared by polyacrylamide gel electrophoresis. The enzyme reaction was continued at 35 °C for 22 h in 2.7% (w/v), pH 6.0 casein solutions. The amount of enzyme added was sufficient to coagulate 100 ml of fresh skim milk in 30 min at 35 °C and pH 6.0. Samples for electrophoresis were taken at intervals.

Caseins prepared from the three kinds of milk gave 11, 12 and 13 protein bands, resp., in the electrophoretic system applied.

The $\varkappa$-fraction of casein I was entirely transformed by CR to para-$\varkappa$-casein within 30 min. With MR the degradation product of $\varkappa$-casein was more strongly shifted to the cathode than in the case of CR. Changes in the electrophoretic behaviour of α_s- and β-caseins were most marked and most different with the two enzymes after 22 h of incubation. With MR the breakdown of β-casein was nearly complete by the end of this period and α_s-casein was split into several faint, fast moving bands. With CR, the main fractions of both α_s- and β-casein were still discernible, although of reduced mobilities.

The decomposition, by either enzyme, of the protein bands in the region of the $\varkappa$-component of casein II occurred in a somewhat different way than with casein I, however, no retardation could be observed. Pasteurization was found to increase the accessibility of α_s- and β-casein to CR, while the action of MR on these proteins was less marked.

With CR as clotting agent, the degradation of the $\varkappa$-fraction of casein III progressed considerably during the first 30 min of the reaction and was complete in 60 min, whereas the action of MR on this component was greatly retarded. During the first 60 min the decomposition of α_s- and β-casein was equally found to proceed at a lower rate with MR. However, after 22 h of incubation, hydrolysis of the above fractions to low molecular weight components was nearly complete, while 15 protein bands were present in the electrophoretogram of casein III treated with CR.

The majority of the results obtained with casein I were in agreement with findings of others. No data were found as to the changes in the electrophoretic behaviour of caseins from pasteurized or hydrogen peroxide-treated milks as caused by different coagulants.

The experiments reported here clearly demonstrate the differences between the actions of the two milk-clotting enzymes. However, these show only after rather prolonged incubation and might play but an inferior role, if any, in cheese-making practice.

During experiments aimed at the utilization, in cheese-making, of a microbial rennet derived from *Endothia parasitica*, hydrogen peroxide – catalase treatment of milk was found to reduce clotting rate as compared to that obtained with calf rennet. Since an earlier study (VÁMOS-VIGYÁZÓ *et al.*,

1978) had revealed distinct differences in the electrophoretic protein patterns of raw, pasteurized and hydrogen peroxide treated milks, it was thought promising to compare, by a similar method, the action of calf rennet and of the microbial coagulant on caseins prepared from above milks. A better knowledge of the mechanism of action of the microbial enzyme might contribute to establish optimum conditions of its utilization.

1. Materials and methods

1.1. Materials

1.1.1. The milk. – Raw skim milk was supplied by a Budapest dairy plant. Pasteurization was carried out at 65 °C during 30 min and was followed by rapid cooling to 20 °C. Hydrogen peroxide treatment as used in this country in *Emmental cheese* production has been described in detail in the paper cited (VÁMOS-VIGYÁZÓ *et al.*, 1978).

1.1.2. Casein. – Casein was obtained from raw (I), pasteurized (II) and hydrogen peroxide treated (III) milk essentially by the method of VANDERPOORTEN and WECKX (1972) with slight modifications as described in the paper cited (VÁMOS-VIGYÁZÓ *et al.*, 1978).

1.1.3. Enzyme preparations. – Crystalline calf rennin (CR) was purchased from SIGMA (U.S.A.). Its clotting activity on raw skim milk was found to be 830,000 SUg^{-1} (SU = *Soxhlet* unit; 1 SU = the amount of milk in ml, coagulated by 1 g or 1 ml of enzyme preparation in 40 min at 35 °C).

The microbial coagulant (MR) was purified from an industrial-scale experimental preparation of the *E. parasitica* enzyme by two gel filtration steps on *Sephadex* G 10 and G 100, resp., and was subsequently concentrated by vacuum evaporation to a specific clotting activity of 240,000 SU per g protein.

For activity measurements 0.5 ml of a suitable dilution of the enzyme preparations were added to 10 ml of fresh skim milk, both preheated to 35 °C.

1.2. Methods

1.2.1. Enzymatic degradation of casein. – The enzyme reaction was carried out in a 2.7% casein solution (w/v) in distilled water, the pH of which was adjusted with 1 *N* NaOH to 6.0 as checked in a *Radiometer* PHM 4 type pH meter. The solution was then filtered. The concentration of the solution corresponded to the casein content of milk.

25 ml of the casein solution preheated to 35 °C were mixed with 1 ml of enzyme solution. The amount of enzyme added was sufficient to coagulate 100 ml of fresh skim milk in 30 min at 35 °C and pH 6.0.

The reaction mixtures were incubated for 22 h at 35 °C and 2 ml samples were taken for analysis, beside of the starting and the end points, after 5, 10, 15, 30 and 60 min. In all the samples taken the reaction was stopped by adding an equal volume of pH 8.6 sodium veronal – HCl buffer (ionic strength 0.005), whereafter the solutions were stored at −20 °C. The enzyme reaction was carried out in triplicate.

1.2.2. Polyacrylamide gel electrophoresis. – 1 ml each of the samples diluted with the veronal buffer were mixed with 1 ml of 45% glycerine and 0.1 ml of bromophenol blue solution. (For this purpose an 0.5% stock solution of the dye in 1% acetic acid was diluted with veronal buffer according to para. 1.2.1 in a ratio of 5 : 95.) 0.2 ml portions of these sample solutions containing $\simeq$1.3 mg of protein were applied per gel tube in an *Acrylophor* (PLEUGER, Belgium) analytical electrophoresis apparatus.

Electrophoresis in 5% polyacrylamide gel containing 0.13% bis-acrylamide and 5 M urea was carried out at pH 8.6 in the veronal buffer according to para 1.2.1, exactly as described earlier (VÁMOS-VIGYÁZÓ *et al.*, 1978). Runs took 45 min at 15 °C (tap-water cooling) and 5 mA per gel and were performed in duplicate.

The displacement of the protein bands stained with *Amido black* according to the paper cited was related to that of bromophenol blue (M_B value). At least two tubes each from two runs (*i.e.* a total of four gels) were taken to calculate relative mobilities.

2. Results

2.1. Degradation of casein from raw milk

Electrophoretic patterns of the protein fractions as obtained from casein I with the two enzymes after different reaction times are shown, along with relative mobilities and their standard deviations, in Fig. 1.

As can be seen from Fig. 1, 11 protein fractions could be detected in casein obtained from raw skim milk. In the order of increasing mobilities fractions No. 1, 6 and 8 corresponded to the bulk of $\varkappa$, α_s- and β-casein, respectively.

The action of CR on $\varkappa$-casein became evident as soon as after 5 min of incubation and some splitting of the β-fraction could be noticed, too. After 30 min, $\varkappa$-casein was entirely transformed to para-$\varkappa$-casein. This manifested itself in a noticeable decrease in mobility of fraction No. 1, on the one hand,

and in the relative invariability of the corresponding values of the bands in the $\varkappa$-zone (Nos. 1–4) between 30 and 60 min of reaction time, on the other. After the extremely prolonged reaction time of 22 h (1320 min) the main para-$\varkappa$-casein band still maintained its former mobility, while the rest of the bands were shifted towards the cathode. Two fractions of those observed after 60 min disappeared.

MR acted on casein in a somewhat different way. The mobility of the main $\varkappa$-band was shifted, after 15 min, more strongly towards the cathode than in the case of CR. Simultaneously with the $\varkappa$-fraction α_s- and, somewhat later β-casein were attacked by the enzyme as well. Changes were most marked and most different from those observed with CR, after 22 h of incubation. By the end of this period breakdown of β-casein by MR was nearly complete and α_s-casein was split into several faint, fast moving bands. With CR the main fractions of both α_s- and β-casein were still present, although of reduced mobilities.

2.2. Degradation of casein from pasteurized milk

Relative migration data and electrophoretic patterns of the protein bands of casein II as well as of their enzymic degradation products are shown in Fig. 2.

The electrophoretogram of casein II contained 12 fractions. The relative mobilities of the bands in the $\varkappa$- and, to a lesser extent, in the β-casein region are somewhat shifted towards the anode as compared to those of casein I. The main band of $\varkappa$-casein ($M_B = 7.9$) is fainter and broader.

The decomposition of the protein bands in the $\varkappa$-casein region by either enzyme occurs in a somewhat different way than with casein from raw skim milk, however, no retardation can be observed. The multitude of fractions appearing in the regions of α_s- and β-caseins after prolonged reaction times with CR indicates that heat treatment increases the accessibility of these proteins to the action of this enzyme. The action of MR on the α_s- and β-bands is definitely less marked and, even after 22 h of incubation, less complete than in the case of raw-milk casein: part of the main bands of these proteins is still present after 22 h of incubation.

It seems that the difference in the action of the two enzymes is less marked on casein prepared from pasteurized as compared to that obtained from raw milk.

2.3. Degradation of casein from hydrogen peroxide–catalase treated milk

The relative mobilities of casein III and its degradation products obtained upon action of the two renneting agents are represented in Fig. 3.

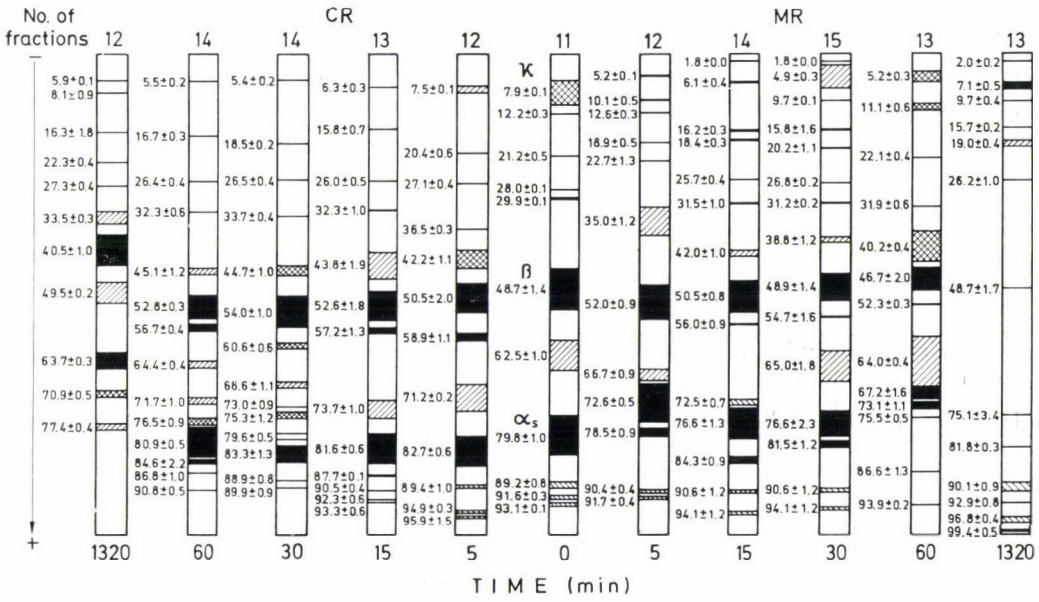

Fig. 1. Electrophoretograms of casein obtained from raw skim milk (casein I) and of the products of its degradation by calf rennin (CR) and a microbial milk clotting enzyme preparation derived from *Endothia parasitica* (MR), resp. Experimental conditions: 25 ml of 2.7% casein solution were incubated at pH 6.0 and 35 °C with 1 ml of enzyme solution for 22 h. Enzyme concentration was selected so as to perform clotting of 100 ml fresh skim milk (pH 6.0, 35 °C) in 30 min. 2-ml samples were taken at the intervals indicated in the Figure. The reaction was stopped by addition of 2 ml Na-veronal–HCl buffer, pH 8.6 (ionic strength 0.005), the solution mixed with an equal volume of glycerine and 1/10 volume of bromophenol blue marker. 0.2 ml portions (≅ 1.3 mg of protein) were applied per gel in a vertical tube gel electrophoresis apparatus (*Acrylophor*, PLEUGER, Belgium). Electrophoresis was carried out in gels containing 5% acrylamide, 0.13% bis-acrylamide and 5 M urea. Runs took 45 min at 15 °C and 5 mA/gel in the buffer indicated above. Staining with Amido black as well as destaining were carried out according to VÁMOS-VIGYÁZÓ *et al.* (1978). Displacement of protein zones was related to that of bromophenol blue. Data represent means obtained from migration values of 4 gel rods belonging to 2 separate electrophoretic runs. α_s, β and $\varkappa$ = main fractions of α_s, β- and $\varkappa$-caseins, resp. The arrow indicates the direction of migration, − and + the cathode and anode, resp. The figures to the left of the electrophoretograms represent means of 4 parallels and standard deviations

Casein obtained from hydrogen peroxide treated milk gave 13 fractions in gel electrophoresis. The relative mobilities of the main bands of β- and $\varkappa$-casein were similar to those of the corresponding fractions of casein I, that of α_s-casein was somewhat lower.

With CR as clotting agent, the degradation of $\varkappa$-casein progressed considerably during the first 30 min of the reaction and was complete in 60 min, whereas the action of the microbial enzyme on this component was greatly retarded. After 60 min $\varkappa$-casein still gave a distinct band and a fraction of reduced mobility corresponding to para-$\varkappa$-casein could be detected only in the 22-h sample.

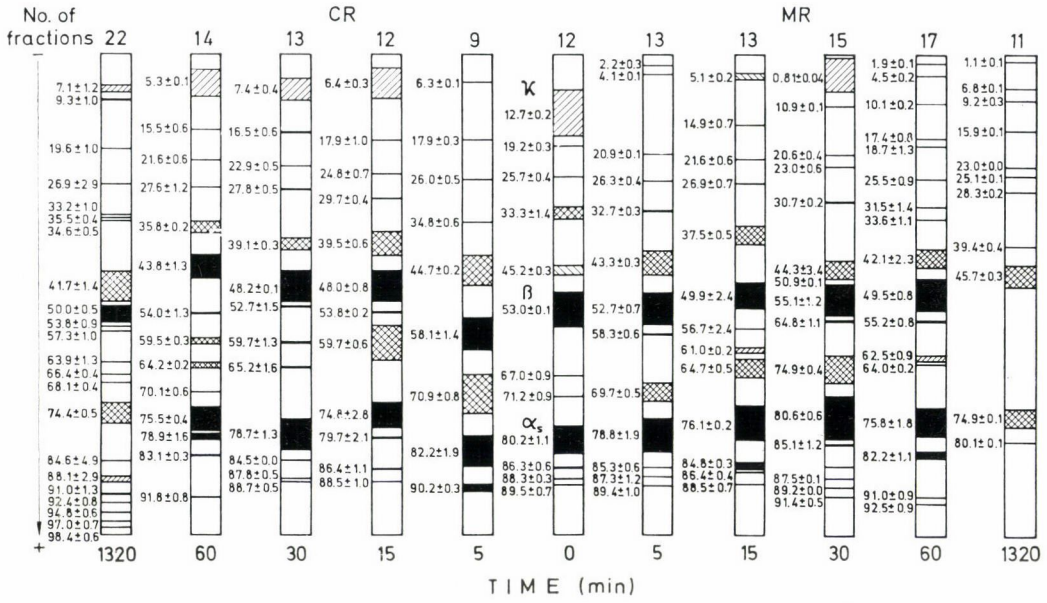

Fig. 2. Electrophoretograms of casein obtained from pasteurized (65 °C, 30 min) skim milk (casein II) and of the products of its degradation by calf rennin (CR) and a microbial milk clotting enzyme preparation derived from *Endothia parasitica* (MR), resp. Experimental conditions and symbols as in Fig. 1

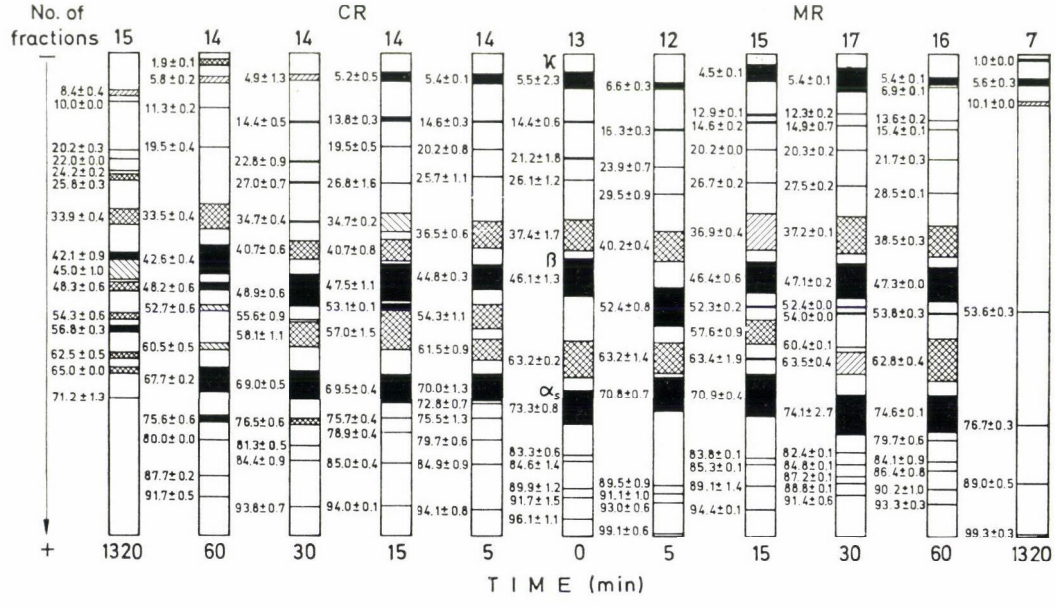

Fig. 3. Electrophoretograms of casein obtained from hydrogen peroxide-treated skim milk (casein III) and of the products of its degradation by calf rennin (CR) and a microbial milk clotting enzyme preparation derived from *Endothia parasitica* (MR), resp. Experimental conditions and symbols as in Fig. 1

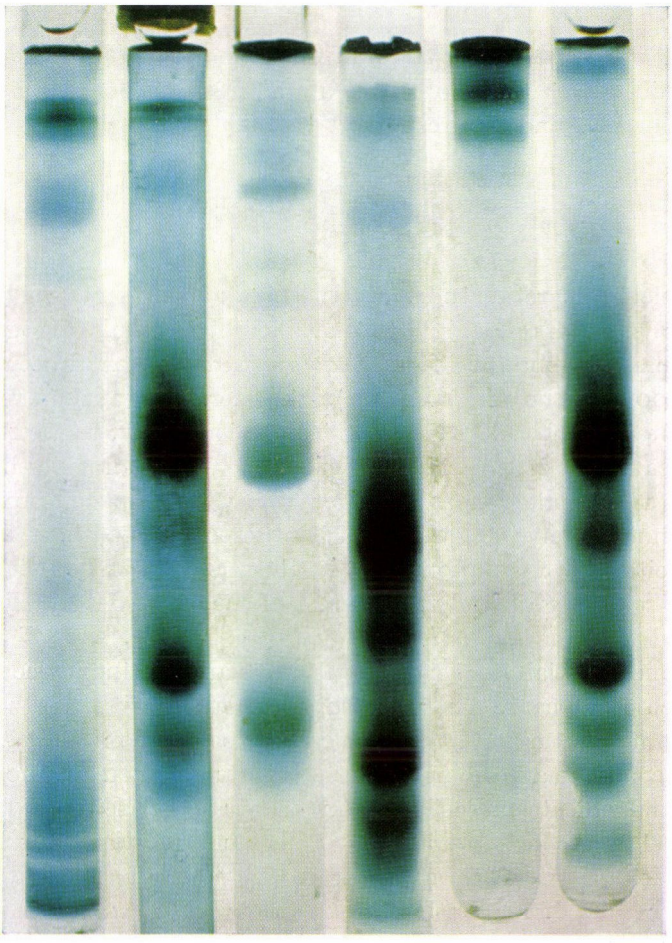

Fig. 4. Protein patterns of caseins obtained from raw (I), pasteurized (II) and hydrogen peroxide (III) treated skim milks after 22-h incubation periods with calf rennin (CR) and a microbial milk clotting enzyme preparation derived from *Endothia parasitica* (MR), resp. Experimental conditions as in Fig. 1. Gels from left to right: 1. casein I + MR, 2. casein I + CR, 3. casein II + MR, 4. casein II + CR, 5. casein III + MR, 6. casein III + CR

During the first 60 min the decomposition of the fractions α_s- and β-casein seems equally to proceed at a lower rate with MR. However, at the final stage, after 22 h of incubation, hydrolysis of the above fractions to low molecular weight components is nearly complete, since hardly any bands can be found that stain with *Amido black*. After the same lapse of time the samples

incubated with CR still contained 15 protein bands. It might be of interest to note that all of these bands had lower mobilities than the main band of α_s-casein.

Fig. 4 gives a good comparison of the differences in electrophoretic patterns as obtained with the two enzymes after 22 h of incubation in the solutions of caseins I, II and III, resp.

3. Conclusions

A gel electrophoretic study into the breakdown of raw-milk-casein and casein fractions by calf rennet and various microbial coagulating agents, among others a preparation obtained from *Endothia parasitica*, had been carried out by VANDERPOORTEN and WECKX (1972). However, these authors reported only on results obtained after 20 to 22 h of incubation. They, too, established the complete breakdown of $\varkappa$-casein by this time, manifesting itself in the appearance of a cathodic band. However, their findings concerning α_s- and β-casein are not entirely in agreement with those presented here. According to the authors cited the α_s-component of casein was ultimately split into 3 fractions by calf rennet, while only the fastest of these was detectable in the pattern obtained with the *Endothia parasitica* preparation. According to Fig. 1, the α_s-component initially consisted of 1 major and 3 minor bands, one minor band of which disappeared by the end of the reaction with CR, while a multitude of fast moving minor bands appeared upon action of MR. The latter finding is in complete agreement with the results reported by MICKELSEN and FISH (1970) and indicate extensive decomposition of α_s-casein. VANDERPOORTEN and WECKX (1972) report on one major additional fraction being formed from the β-component of casein by calf rennet, while no band appeared at all in the β-region after 20 h of incubation with the *Endothia parasitica* preparation. According to Fig. 1 a new major band of the β-component just ahead of the main one appears at an earlier stage of the reaction with CR and gets somewhat fainter by the end of the observation period. In the case of MR a faint band of the main β-component still remains after 22 h of incubation. The results with respect to the decomposition of α_s- and β-caseins from raw milk as described here are borne out by findings of LEDFORD and co-workers (1968) as well as of TAM and WHITAKER (1972). These authors found in electrophoretic studies a more complete hydrolysis of the above proteins with the *Endothia parasitica* enzyme than with calf rennet, whereby α_s-casein proved more susceptible to the action of both coagulants than β-casein and the latter less resistant towards the microbial preparation than towards rennin.

In the present study both CR and MR were purified enzymes, while the authors cited used commercial preparations or, as in the case of VANDERPOOR-

TEN and WECKX (1972), preparations of unspecified degrees of purity. The complete disappearance of the β-component after 20 h of incubation with the microbial enzyme as described by the above authors suggests that they, too, used commercial grade preparations containing impurities of non-specific proteolytic activity.

In spite of some differences as discussed above the results described in the present paper confirm the findings of the authors cited according to which degradation of the main casein components by animal and microbial rennets occur in different ways. This, however, manifests itself distinctly only after rather prolonged reaction times never occurring in practice.

No data were found as to the changes in the electrophoretic behaviour of caseins from pasteurized and hydrogen peroxide-treated milks as caused by different coagulants.

According to the results obtained pasteurization reduced the differences between the actions of the two enzymes (Fig. 2). This might be of practical importance in cheese-making, since most cheeses are manufactured from heat treated milks.

The results obtained with hydrogen peroxide treated milk (Fig. 3) indicate that retarded clotting of milk as observed with MR is due to delayed degradation of $\varkappa$-casein. This might be caused by structural modifications, although, as reported by FISH and MICKELSEN (1967) these are not apparent from the electrophoretograms. Investigations into the nature of these supposed modifications are in progress. The hydrogen peroxide treatment of milk has a retardatory effect also on the α_s- and β-casein decomposing capacity of MR, but only during the first 60 min of the reaction. On prolonged incubation the non-specific proteolytic activity of this enzyme (which, even in a highly purified state is several times higher than that of rennin) (KISS *et al.*, 1976), increases and leads to almost complete hydrolysis of these proteins. Fortunately, this cannot occur in cheese-making, since clotting times are relatively short (30 min) and during the subsequent technological operations as well as in cheese-ripening the proteolytic activity of the renneting agent probably plays but an inferior role as compared to the more complex action of starter bacteria. Thus it is possible to obtain, with the two enzymes, cheeses of very similar chemical composition and sensory properties (KISS *et al.*, 1975; VÁMOS-VIGYÁZÓ *et al.*, 1975).

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A STUDY INTO SOME PROPERTIES OF PEROXIDASE IN VEGETABLES

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Some characteristics of peroxidase as present in kohlrabi and cauliflower were compared under identical experimental conditions. Enzyme activity was determined according to a method described earlier (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975) and was expressed in terms of specific activity (= activity related to the mass, wet-weight basis).

The specific enzyme activity was distributed, in the parts of kohlrabi, in parallel to solids content, while in cauliflower it varied in the opposite sense.

The pH dependence of specific enzyme activity was similar in both products: the highest values were measured in the pH-range between 5.1 and 5.9 with actual (non-significant) maxima at pH 5.7 and 5.5, resp. Specific peroxidase activity increased with temperature in both products up to 40 °C. The apparent activation energy of the reaction catalyzed by the enzyme in kohlrabi was about double (40.6 kJ mol^{-1}) the value obtained with cauliflower homogenate (20.9 kJ mol^{-1}). For the descending section of the relationship between activity of cauliflower peroxidase and temperature, the activation energy was 63.3 kJ mol^{-1}.

Ten-min heat treatments at various temperatures in the range of 55 to 120 °C had different effects on peroxidase in the two products: the enzyme in cauliflower was inactivated at 95 °C, while that in kohlrabi retained 0.1% of its original activity even at 120 °C. The shapes of the curves describing the relationships between percentage residual activity and temperature of heat treatment were different, too: for peroxidase in kohlrabi the best approximation could be achieved by a logistic, and for that in cauliflower by a linear function. Similarly to heat resistance, the enzyme in kohlrabi showed also a higher regeneration capacity: after a 10-min treatment at 90 °C, residual activity rose within 24 h at 25 °C from 2.2% to 28.2%, while in the case of cauliflower the corresponding values were 0.05% and 0.14%. The time course of regeneration suggests a dependence on the temperature of heat treatment or, rather, on the degree of inactivation. Investigations into this direction have been considered.

Freezing and storage in the frozen state decreased the specific peroxidase activity in both products, whereby the enzyme of kohlrabi was affected to a lesser extent.

The results indicate that, except the pH dependence and the temperature optimum of activity, peroxidases in kohlrabi and cauliflower are distinctly different.

The unfavourable changes in flavour, colour and consistency occurring during storage of processed horticultural products are attributed mainly to enzymes. As a means of prevention, processing generally includes a step of enzyme inactivation most often carried out by heat treatment or by a combined procedure involving heating and another method of preservation. Among the enzymes present in horticultural products peroxidase is of outstanding heat stability, especially in vegetables of less acidic pH. Earlier studies (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975, 1976) have shown concentration and heat resistance of

peroxidases as found in various vegetables to be rather different. Heat stable peroxidases with a tendency to regeneration after thermal treatment were found, among others, in kohlrabi (VÁMOS-VIGYÁZÓ *et al.*, 1978, 1979) and cauliflower. Since in the vast literature dealing with peroxidases information available on the enzymes of these products is very scarce, more detailed investigations into their behaviour were thought to be of interest.

The present paper gives a comparison of some properties of peroxidase as found in homogenates of kohlrabi and cauliflower, resp.

1. Materials and methods

1.1. The vegetables

Kohlrabi *(Brassica oleracea var. gongyloides)* and cauliflower *(Brassica oleracea var. cauliflora)* of the cvs. "*Kék szalonna*" (Blue Lard) and "*Iglosena*", resp., were both supplied by a local cooperative farm. Both products were stored until utilization at 5 °C in normal atmosphere.

The peeled kohlrabi tuber as well as the cauliflower detached from leaves and stalk were cut into small pieces. For all the experiments with the exception of freezing the comminuted vegetables were subsequently homogenized in an *Ultra-Turrax* blendor using pH 4.0 acetate buffer or, in the heat inactivation experiments, distilled water as medium (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975).

1.2. Measurement of peroxidase activity

Peroxidase activity was determined, according to a method adapted from the literature (WINTER, 1968), at 25 °C and pH 5.0, whereby the reaction mixtures contained 7.4 mM H_2O_2 as substrate and 0.083% o-phenylene diamine as hydrogen donor. Substrate transformation was followed by measuring, at intervals, the optical density (OD) of the coloured reaction products. Readings were taken at 420 nm and enzyme activity was calculated from the linear section of the plots of OD vs. reaction time. 1 Δ OD min^{-1} was considered 1 kU of enzyme activity (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975). In order to facilitate the comparison of enzyme activities as present in various parts of the vegetables, values were related to solids content. In all the other cases activities were related to the mass of the product (wet-weight basis). Both values will be referred to as "specific enzyme activity".

1.3. Investigations into some factors affecting specific enzyme activity

In order to study the relationship of specific enzyme activity and pH, the values of the latter were varied from 3.6 to 7.9 and from 3.2 to 8.0 for

kohlrabi and cauliflower, resp. The influence of temperature was established by activity measurements in the ranges of 15 to 50 °C and 12 to 60 °C, resp., for the two kinds of vegetables, in the order mentioned.

Enzyme inactivation experiments were carried out by 10-min heat treatments at various temperatures and residual activities were determined immediately after, in the samples cooled to 25 °C (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1976). The regeneration of the enzyme was followed by measuring activities, at intervals, in the samples of the heat-treated vegetable homogenates kept at room temperature for 24 h.

In order to study the effect of storage in the frozen state, peeled and sliced kohlrabi as well as cauliflower cut to pieces were put into a −20 °C deep-freezer. Activities were determined in thawed samples after 1 month and, in the case of kohlrabi, also after 5 months of storage.

2. Results

2.1. The distribution of peroxidase activity in various parts of the products

Peroxidase activities as related to solids content of the various parts of the vegetables are shown in Fig. 1.

In kohlrabi, specific peroxidase activity was highest on the root side, this was followed by the central part and the peel, the lowest value having been found on the shoot side. The ratios of the specific activities were, in the above order, 2.8 : 1.9 : 1.6 : 1, while those of solids content were 1.3 : 1.05 : 1.02 : 1. The variations of the solids content within the product were much slighter than those of the specific activity, however, their tendency was the same.

The specific peroxidase activity of cauliflower was found to be higher in the "stem" that connects the "flower" with the stalk, the ratio of the two values being 1.7 : 1. The ratio of the solids contents was 0.89 : 1, *i.e.* – in contrast to kohlrabi – there was no parallelism between these two characteristics.

For further studies homogenates prepared from average samples of the products were used.

2.2. Changes in specific peroxidase activity with pH

The dependence on pH of the specific peroxidase activities of the two vegetables is illustrated in Fig. 2.

The maxima of specific peroxidase activity for kohlrabi and cauliflower were found at pH 5.7 and 5.5, resp., *i.e.*, the two values were very near to each other. The maxima of specific activity were not significantly different from

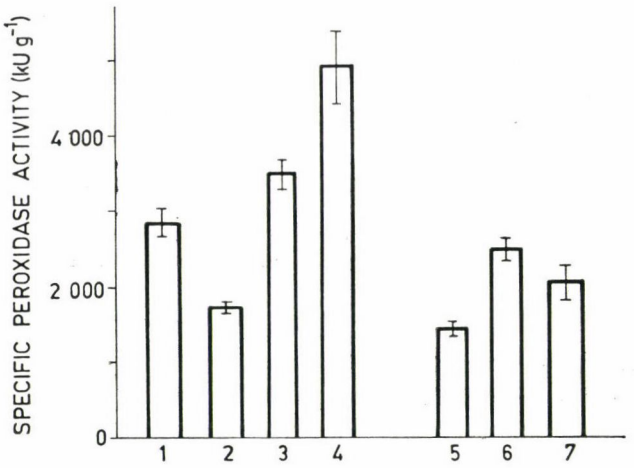

Fig. 1. Distribution of peroxidase activities as related to solids contents in the various parts of kohlrabi and cauliflower. *1–4:* kohlrabi, cv. *Kék szalonna* (Blue lard); *1:* peels, *2:* shoot side, *3:* central part, *4:* root side, *5–7:* cauliflower, cv. *Iglosena; 5:* "flower", *6:* "stem", *7:* average sample. The vertical bars represent standard deviations (2s). The number of parallel determinations, n = 3 for kohlrabi and n = 6 for cauliflower

the other values established in the pH ranges from 5.2 to 5.9 for kohlrabi and from 5.1 to 5.7 for cauliflower. At both ends of the pH range investigated, specific peroxidase activities in both vegetables dropped to about 10% of the maximum values.

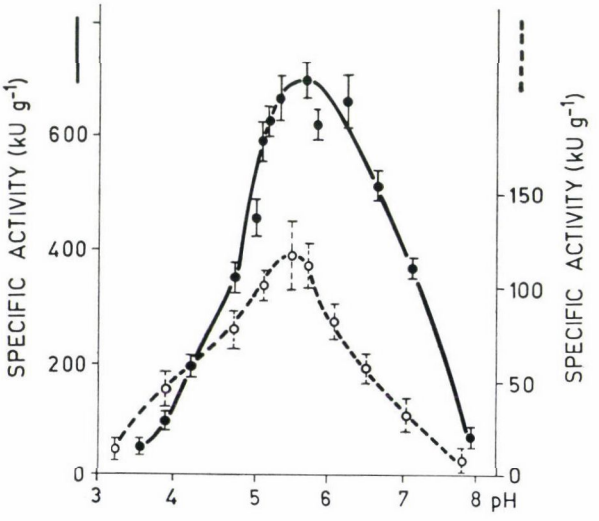

Fig. 2. Changes in specific peroxidase activity with pH. Kohlrabi: full line (n = 3), cauliflower: broken line (n = 6). The vertical bars represent standard deviations (2s)

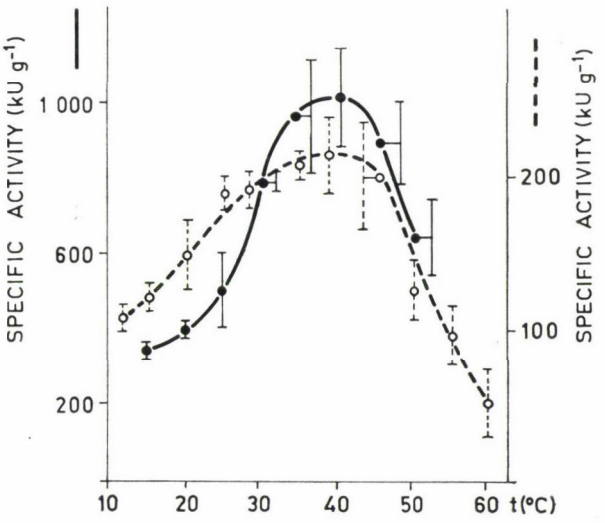

Fig. 3. The dependence of specific peroxidase activity on temperature. Kohlrabi: full line (n = 3), cauliflower: broken line (n = 6). The vertical bars represent standard deviations (2s); t = temperature (°C)

2.3. Changes in specific peroxidase activity with temperature

The changes in specific peroxidase activity as observed in the temperature ranges investigated are shown for both products in Fig. 3.

In both kinds of vegetables specific activity was found to increase with temperature up to 40 °C and to decrease thereafter. Owing to the relatively high standard deviations the decrease was, however, not significant until 50 °C. The ascending sections of the curves may be described fairly well by the *Arrhenius* equation, as can be seen in Fig. 4.

The relationship between the logarithm of specific activity and the reciprocal absolute temperature was significant, for both products, at a probability level of 99%. The apparent activation energy of the reaction catalyzed by peroxidase as calculated from the slope of the regression curve was about twice as high for kohlrabi (40.6 kJ mol^{-1}) as for cauliflower (21.9 kJ mol^{-1}). In the case of the latter, similar calculation yielded for the descending section of the curve in Fig. 3 an activation energy of 63.3 kJ mol^{-1}. For kohlrabi this calculation could not be performed, owing to the insufficiency of data in the descending section.

2.4. Inactivation of peroxidase by heat treatment

The specific peroxidase activities of the kohlrabi and cauliflower homogenates kept for 10 min at various temperatures and then quickly cooled to 25 °C are expressed as percentage of the values of untreated samples in Fig. 5.

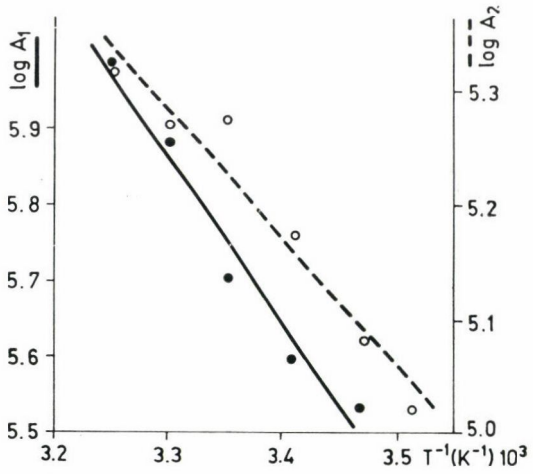

Fig. 4. The relationship between the logarithm of specific peroxidase activity and reciprocal absolute temperature. A_1 and A_2: specific activities (kUg^{-1}) of peroxidase in kohlrabi and cauliflower, resp. Kohlrabi: full line and solid circles, cauliflower: broken line and open circles. T = temperature (K). Regression equations of the curves:

$\log A_1 = 12.88\text{–}2.13 \cdot 10^3\, T^{-1}$; $r^2 = 0.96^{**}$; $N = 5$;

$\log A_2 = 9.07\text{–}1.15 \cdot 10^3\, T^{-1}$; $r^2 = 0.95^{**}$; $N = 6$.

Apparent activation energies (E_{a_1} app. and E_{a_2} app.) for kohlrabi and cauliflower peroxidase, resp.: E_{a_1} app. = 40.6 kJ mol^{-1}, E_{a_2} app. = 21.9 kJ mol^{-1}; r^2 = coefficient of determination; N = number of points of measurement

** = The relationship is significant at the probability level of 99%

The peroxidases of the two products show marked differences in heat resistance: while a 10-min exposure to 55 °C did not cause any significant change in the specific activity of the kohlrabi enzyme as compared to measurements at 25 °C, it decreased the corresponding value for cauliflower by 20%. After a heat treatment of the same duration at 95 °C, activity in the latter vegetable was below the limit of measurability, whereas in kohlrabi peroxidase still maintained 0.6% of its original value. Even a 10-min exposure to 120 °C was not sufficient to destroy entirely peroxidase activity in the latter product: 0.1% of the original value survived the treatment.

In the case of kohlrabi, the changes in specific peroxidase activity as they occur upon 10-min thermal treatment in the temperature range of 55 to 120 °C could be best fitted to a logistic relationship. With cauliflower the relationship between residual activity and temperature of thermal treatment could be described, in the range of 55 to 90 °C, with excellent approximation ($r^2 = 0.98$), by a linear equation. Fig. 5 shows the points of measurement and the calculated curves for the enzymes of both products.

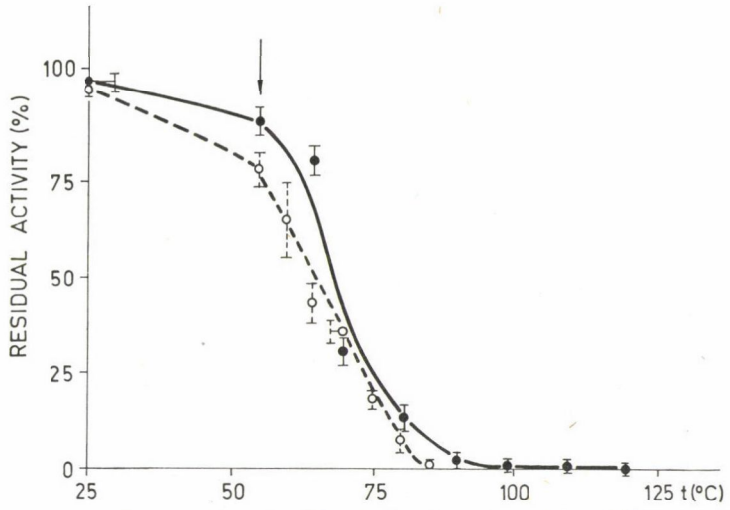

Fig. 5. Inactivation of peroxidase by 10-min heat treatments at different temperatures. Kohlrabi: full line, cauliflower: broken line. The number of parallel activity determinations, n = 3 and 6, resp. The vertical bars represent standard deviations (2s). t = temperature (°C)

Regression equations of the curves:

$$RA_1 = 100 - 100\left(\frac{1}{1+e^{13.42-0.196t}}\right); \qquad RA_2 = 226.71 - 2.73\,t$$

$r^2 = 0.96$***; $r^2 = 0.98$***;
$N = 8$; $N = 7$;
$t_r = 55$–120 °C; $t_r = 55$–90 °C.

RA_1 = residual activity in kohlrabi; RA_2 = residual activity in cauliflower (both: % of initial); t = temperature, °C; r^2 = coefficient of determination; *** = the relationship is significant at the probability level of 99.9%. N = number of points of measurement, t_r = temperature range taken into consideration in calculating the equations. The arrow indicates the beginning of this range

2.5. Regeneration of peroxidase after heat treatment

The changes in specific peroxidase activity of kohlrabi homogenates exposed for 10 min to 90 °C and 120 °C, resp., as well as of cauliflower homogenates heated for the same time at 90 °C were investigated at intervals during the 24-h period (at room temperature) that followed heat treatment. The results are shown in Fig. 6.

Immediately after heat treatment at 90 °C ("0" time), residual peroxidase activity in kohlrabi was 2.2% of the original value and this increased in 24 h at room temperature to 28.2%. The respective values for the heat treatment at 120 °C were 0.02% and 0.77%, and for the 90 °C treatment of the cauliflower homogenate 0.05% and 0.14%.

In kohlrabi exposed to 90 °C the greater part of the activity was restored *during* the first 2 h that followed heat treatment, while in the kohlrabi sample exposed to 120 °C and in cauliflower kept at 90 °C regeneration took place mainly *after* the first 2 h.

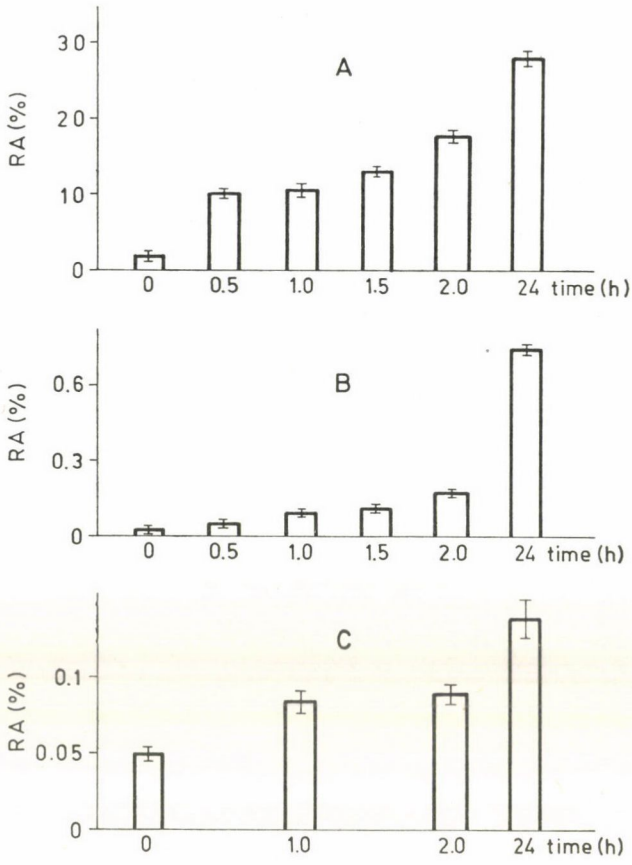

Fig. 6. Reactivation of peroxidase at room temperature after a 10-min heat treatment at different temperatures. A: kohlrabi exposed to 90 °C; B: kohlrabi exposed to 120 °C; C: cauliflower exposed to 90 °C. *RA:* residual specific activity as related to the value found in the untreated sample; t = reactivation time after heat treatment (room temperature). The number of parallel determinations, n = 3 for kohlrabi, n = 6 for cauliflower. The vertical bars represent standard deviations

In cauliflower homogenates kept for 10 min at 95 °C no regeneration was observed within 24 h.

2.6. Changes in peroxidase activity during frozen storage

Specific peroxidase activities of thawed kohlrabi stored at −20 °C for 1 and 5 months, resp., as well as of cauliflower stored under similar conditions for 1 month can be seen in Fig. 7.

Kohlrabi peroxidase proved much more resistant towards frozen storage than the enzyme of cauliflower: after 1 month of frozen storage, specific activity of the former product dropped by about 20% only as compared to

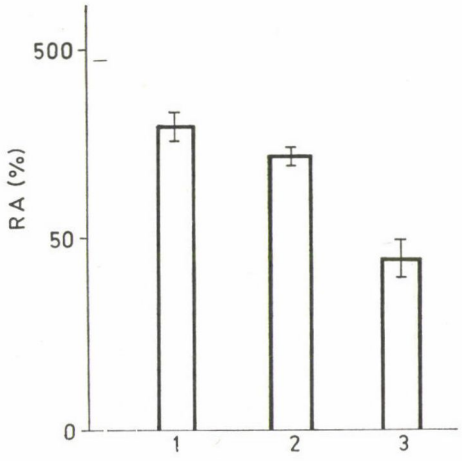

Fig. 7. Changes in specific peroxidase activity during storage at −20 °C. *RA:* residual activity after thawing as related to the original value determined in the raw sample prior to freezing. *1:* kohlrabi stored for 1 month, *2:* kohlrabi stored for 5 months, *3:* cauliflower stored for 1 month. The number of parallel determinations, n = 3 for kohlrabi, n = 6 for cauliflower. The vertical bars represent standard deviations

the value found in the raw untreated sample, while in the latter the loss amounted to about 50%. Even after 5 months of frozen storage, about 70% of the original specific peroxidase activity were still present in frozen kohlrabi.

3. Conclusions

Peroxidases in kohlrabi and cauliflower were found to be very similar with respect to the pH- and temperature-dependences of activity, and fairly different in resistance towards heat treatment and frozen storage.

Specific peroxidase activities were highest for both products in the pH range of 5.1 to 5.9. In an earlier study the pH optimum of peroxidase activity in potato homogenates as determined under similar conditions was found to be in the range of pH values between 5.1 and 5.7 (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975). This finding fits in well with the results presented here. According to the literature (REED, 1975) the pH optimum of peroxidase activity depends mainly on the nature of the (donor) substrate.

The temperature dependence of specific peroxidase activity was found to be different for potato homogenates (MIHÁLYI & VÁMOS-VIGYÁZÓ, 1975) as compared to the vegetables dealt with in the present study. This might be due to the influence of the natural environment (PARK *et al.*, 1977; ADAMS, 1978), *e.g.* to cell constituents that might increase or decrease heat stability.

The activation energy of the inactivation process of purified horseradish peroxidase was found to be 88 kJ mol^{-1} for the heat stable and 142 kJ mol^{-1}

for the thermolabile isoenzyme fraction (LING & LUND, 1978). The value obtained in this study for the inactivation of the enzyme in cauliflower was considerably lower (63 kJ mol^{-1}), possibly due, at least partly, to the influence of the natural environment. Further factors that might cause the difference are, among others: different isoenzyme composition or different hydrogen donors used in the assay (ZOUEIL & ESSELEN, 1959).

The differences in the shape of the curves obtained for the two products on plotting residual activities against the temperature of thermal treatment indicate considerable differences in heat stability of the respective individual isoenzymes. Investigations into this problem are in progress and have been partly dealt with elsewhere (VÁMOS-VIGYÁZÓ *et al.*, 1978).

The dependence of the course of enzyme reactivation on the temperature of heat treatment has, as to our knowledge, not been mentioned so far in the literature. Further work to clear this point has been considered.

Frozen storage was found to decrease the specific peroxidase activity in both products, whereby the enzyme of kohlrabi proved to be much more resistant. The only reference found in the literature as to the effect of freezing on peroxidase (BURNETTE, 1977) is in striking contrast with these findings by stating that activity in kohlrabi was stimulated eightfold by freezing. Unfortunately, the details of these experiments are not known.

Summarizing the results of the study presented it might be said that under identical experimental conditions the peroxidase enzymes as found in the non-acid vegetables kohlrabi and cauliflower differed mainly in heat stability (including regeneration capacity after thermal treatment) and resistance towards freezing and storage in the frozen state. Further studies will, therefore, be focussed on these topics, the more so as both are of outstanding practical importance.

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INFLUENCE OF THE CULTIVATION TEMPERATURE ON PROTEASE ACTIVITY AND TTC REDUCING CAPACITY OF YEASTS

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Protein content, proteolytic activity and TTC reducing capacity of a psychrophilic and a mesophilic yeast cultured at optimal and suboptimal temperatures were studied. Plotting the proteins of yeasts against their molecular weights it was found that the protein existed in a more disaggregated form if they had been cultured at suboptimal temperatures. Maximum enzyme activities were also found in proteins of lower molecular weight in yeasts cultured at suboptimal temperatures.

The different thermosensitivity of various microorganisms is a well-known phenomenon. A considerable number of experiments were performed to determine amino acid composition (CRAVERI *et al.*, 1973), DNA composition (IRWIN *et al.*, 1973; YAMAGUCHI, 1967) and ribosomal parameters (IRWIN *et al.*, 1973; FRIEDMAN, 1968; PACE & CAMPBELL, 1967) at different cultivation temperatures. In spite of this fact our knowledge is very poor concerning the mechanisms and chemical characteristics of psychrophilic microorganisms accomodating to low temperatures. Thus, we have no data about possible changes in the distribution of cytoplasmic proteins and enzyme activities in microorganisms cultured at different temperatures.

In the present work we report data obtained by determining the molecular weight distribution of cytoplasmic proteins, cytoplasmic protease and TTC reducing activity of a mesophilic and a psychrophilic yeast strain cultured at different temperatures.

1. Materials and methods

1.1. Strains

The mesophilic *Torulopsis utilis* (T_{82}) identified by and obtained from ALKO RESEARCH LABORATORIES, Finland and a psychrophilic *Candida utilis* strain (C_9), isolated by us from the river Danube were used.

1.2. Propagation and maintenance of strains

The cultures were propagated in a medium containing: KH_2PO_4 0.2 g K_2HPO_4 0.15 g, $NaH_2PO_4 \cdot H_2O$ 2.0 g, Na_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g,

$ZnSO_4$ 0.1 g, $(NH_4)_2SO_4$ 3.0 g, Beef extract (DIFCO) 5.0 g, Bacto pepton 4.0 g, Yeast extract (OXOID L21) 1.0 g, glucose (a.p.) 15.0 g, $FeCl_3 \cdot 6H_2O$ 0.1 mg, $CuSO_4 \cdot 5H_2O$ 0.6 mg, $MnCl_2 \cdot 4H_2O$ 0.15 mg, KI 0.05 mg, HBO_3 0.1 mg, in 1 liter medium.

T_{82} was cultured at 37 °C and 25 °C, C_9 at 25 °C and 5 °C, resp., in 500 ml Erlenmeyer flasks at pH 7. The cultures were shaken in an excentric shaker (120–130 strokes min^{-1}). The maintenance of strains was carried out in the same medium completed with 15 g DIFCO Bacto agar/liter.

1.3. Growth characteristics of the strains at different temperatures

The optimal cultivation temperature of C_9 proved to be 25 °C, it grows at 5 °C to visible colonies within 7 days, but does not grow over 30 °C (EDDY, 1960). Thus, C_9 can be considered as a psychrophilic strain.

T_{82} grows well at 30–40 °C, and does not grow below 10 °C. It can be considered as a mesophilic strain.

1.4. Disintegration of cells

At the end of fermentation (maximum cell count) cells were harvested by centrifugation at $6000 \times g$, for 30 min at 5 °C. The biomass obtained was washed twice with 0.75% NaCl solution. The washed pellet was suspended in the same solution (10^9/ml cell count). Disintegration was carried out by a MSE MF. 20. type 60 W ultrasonic disintegrator at 1.5 A current for 3×10 min, in an ice-bath, then the suspension obtained was centrifuged again at $6000 \times g$ for 30 min. The supernatant contains the intracellular proteases.

1.5. Separation of cytoplasmic proteins by gel-filtration

Sephadex G200 (PHARMACIA FINE CHEMICALS, Sweden) was allowed to swell for 5 hours in physiological NaCl at 90 °C and packed into columns SR 25/45. Columns were equilibrated overnight with 1–2 liters of 0.05 *N* CH_3COOH-CH_3COONa buffer for separating proteases and with 0.05 m TRIS buffer for the study of TTC recuding capacity. The void volume of the columns was determined with 2 ml of 0.2% Dextran Blue 2000. Two ml of disintegrated cell supernatants were placed on top of the gel and eluted downwards with the respective buffers at the rate of 4–5 ml/10 min. The effluent was collected in 4–6 ml samples, and the optical density was measured at 280 and 260 nm.

1.6. Assay of proteolytic activity

Bovine Serum Albumine (BSA V., SIGMA) was used as substrate for the determination of proteolytic activity. 2.62 g BSA were dissolved in 10 ml distilled water by stirring with a magnetic stirrer.

74 ml H_2O, 6 ml of 1 *N* NaOH and 36 g urea were added to 10 ml BSA solution. It was kept at room temperature for an hour, then 10 ml of 1 *M* KH_2PO_4 and 4 g urea were added. pH was adjusted to 5.5 with HCl. One mg merthiolate was given to 50 ml substrate, and stored at 5 °C.

One ml of each sample was added to 1 ml of substrate solution. The mixture was incubated at 37 °C in water bath for 18 hours. After adding of 1 ml 0.5 m trichloroacetic acid (TCA) it was homogenized thoroughly.

The precipitate was removed by filtration through Whatman No. 1. paper and the proteolytic activity was measured by the *Lowry* method (LOWRY *et al.*, 1951). The optical density was measured at 660 nm in a UNICAM SP 1800 spectrophotometer after 5 min incubation time. Activity of the samples was expressed in picokatals.

1.7. Determination of TTC reducing capacity

Samples collected for the analysis of TTC reducing capacity were adjusted to 5 ml with 0.5 m TRIS buffer. 0.5 ml 0.5% TTC was added to each sample and the tubes were incubated at the cultivation temperature for 24 hours and 48 hours, respectively.

The water insoluble formazan produced was pelleted and dissolved in 8 ml ethylalcohol (96%), then optical density was determined at 480 nm. Activity was expressed in picokatals.

2. Results and conclusions

Protein contents, intracellular protease activity and TTC reducing capacity of the eluate fractions of the two yeasts used and cultured at optimal and suboptimal temperatures were plotted against the logarithm of molecular weight (Figs. 1–4). All the curves showing the protein contents of the fractions proved to be bimodal and trimodal.

If both of the yeasts were cultured at suboptimal temperatures, a shift toward the proteins of lower molecular weight could be observed.

We could find protease activity only in proteins with molecular weights 1×10^5–4×10^5. Here again, in fractions of cell extracts cultured at suboptimal temperatures the maximum of protease activity was shown in proteins of lower molecular weight. This is also demonstrated in Table 1.

TTC capacity could be observed in proteins of 5×10^3–3×10^4 and 1×10^5–4×10^5 molecular weight.

Culturing the yeasts at suboptimal temperatures, the same tendency as in the case of protease activity, namely shifting the maxima of TTC reducing capacity toward the proteins of lower molecular weight, could be seen. Table 1 clearly shows this tendency, too.

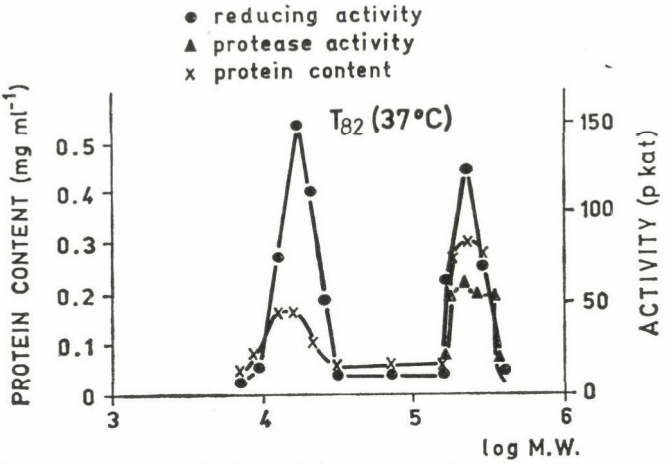

Fig. 1. Protein content, proteolytic activity and TTC reducing capacity of the mesophilic strain (T_{82}) cultured at 37 °C as plotted against the log M.W. of the proteins

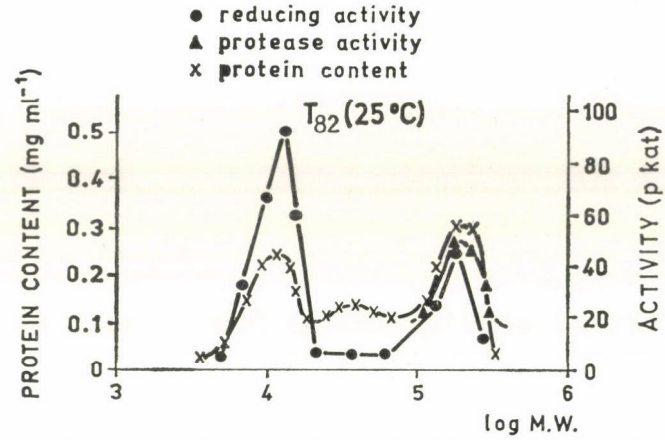

Fig. 2. Protein content, proteolytic activity and TTC reducing capacity of the mesophilic strain (T_{82}) cultured at 25 °C as plotted against the log M.W. of the proteins

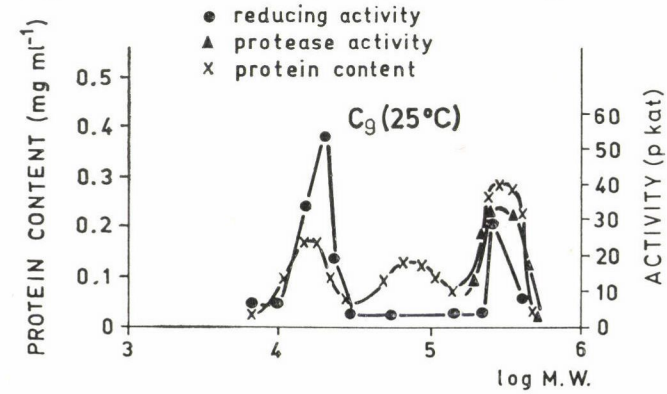

Fig. 3. Protein content, proteolytic activity and TTC reducing capacity of the psychrophilic strain (C_9) cultured at 25 °C as plotted against the log M.W. of the proteins

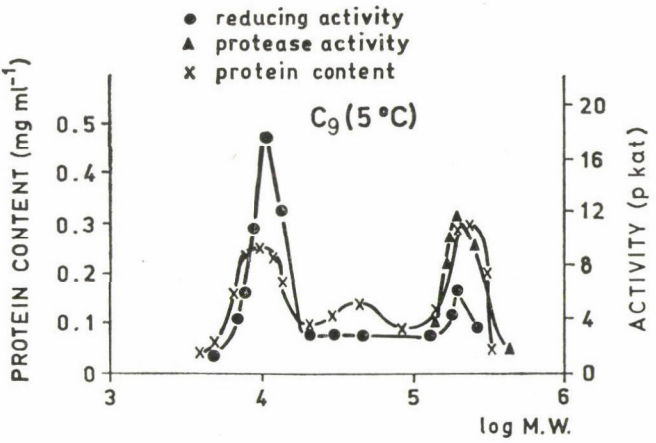

Fig. 4. Protein content, proteolytic activity and TTC reducing capacity of the psychrophilic strain (C_9) cultured at 5 °C as plotted against the log M.W. of the proteins

Table I

Molecular weights of protein fractions showing maximum protease activity and TTC reducing capacity of the mesophilic (T_{82}) and psychrophilic (C_9) strains

Symbols of the strains and cultivation temperature	Molecular weight at maximum protease activity	Molecular weight at maximum TTC reducing capacity	
	x 10^5	x 10^5	x 10^4
T_{82} (37 °C)	1.75	1.90	1.70
T_{82} (25 °C)	1.58	1.68	1.25
C_9 (25 °C)	1.97	2.10	1.90
C_9 (5 °C)	1.60	1.60	1.08

On the basis of the results obtained it can be concluded that proteins of both mesophilic and psychrophilic yeasts cultured at suboptimal temperatures are in a more disaggregated form than those of yeasts cultured at the optimal temperature. This phenomenon may have a role in the adaptation to lower temperatures. The finding that the maximum enzyme activities of the psychrophilic yeast cultured at 5 °C were found approximately in the same molecular weight range as those of the mesophilic strain cultured at 25 °C also indicates an adaptation of higher degree of psychrophilic yeasts to lower temperatures.

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CHANGES INDUCED BY CHYMOSIN AND A MICROBIAL COAGULANT IN KAPPA- AND WHOLE CASEIN

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Differences in the action of chymosin (calf rennin = CR) and a microbial coagulant from *Endothia parasitica* (MR) on pH 6.0 solutions of whole casein and of κ-casein from fresh (I), pasteurized (65 °C, 30 min = II) and hydrogen peroxide treated (III) cow's milk were followed (a) by measurement of non-protein nitrogen (NPN) formed from these as well as (b) by polyacrylamide gel electrophoresis (PAGE) of the latter protein at various stages of the enzyme reaction carried out at 35 °C.

Rate and extent of NPN-formation from whole casein were higher with MR than with CR, although the release of low molecular weight products from caseins II and III started, with the former enzyme, after a 5-min lag period. Pasteurization seemed to reduce and hydrogen peroxide treatment to enhance the proteolytic activity of MR, while either of these processes showed but a slighter effect on NPN-formation by CR.

NPN-release from κ-caseins I and II was essentially similar in trend to the results obtained with whole casein. For κ-casein III findings were markedly different: NPN-formation was found to proceed at a similar rate and to reach, by the end of the observation period (60 min), the same value with both enzymes.

The results of the electrophoretic investigation of κ-casein degradation were in agreement with previous findings established on whole casein substrates. The breakdown of κ-casein I was achieved by both enzymes within the first 5 min of the reaction. However, during the following period, strong deviations were found in the action of the two enzymes, apparently owing to the higher non-specific proteolytic activity of MR. With κ-casein II, the beginning of the reaction seemed to be somewhat delayed in the case of both enzymes. As the reaction progressed, differences in the protein patterns as obtained with the two enzymes increased.

Para-κ-casein formation from κ-casein III was also delayed with both enzymes as compared to κ-casein I, but particularly with MR: in this case the original protein seemed to be partly preserved up to 60 min. In the later phases of the reaction the difference in the mode of action of the two enzymes became strikingly apparent: the protein patterns of κ-casein III treated with CR contained, even after 22 h of incubation, 3 zones of low and medium mobilities, whereas by this time the electrophoretograms obtained with MR showed only two faint bands.

Another paper (VÁMOS-VIGYÁZÓ *et al.*, 1980) gave an account on a comparative electrophoretic study into the actions of crystalline calf chymosin (CR) and of a microbial coagulant (MR) derived from *Endothia parasitica*, using whole caseins prepared from variously treated milks as substrates. The present paper deals with the results of similar experiments carried out with κ-caseins as well as with the comparison of the amounts of non-protein nitrogen (NPN) formed by the two enzymes from these compounds and from whole caseins prepared from fresh (I), pasteurized (II) and hydrogen peroxide treated (III) milks.

1. Materials and methods

1.1. Materials

The milks, whole caseins and enzyme preparations were the same as used in the paper cited. For the experiments with $\varkappa$-caseins MR was purified, after gel filtration, twice on DEAE cellulose (KISS *et al.*, 1976).

$\varkappa$-casein was prepared essentially according to HILL (1963), omitting the chromatographic step (VÁMOS-VIGYÁZÓ *et al.*, 1978) and applying urea for further purification according to ZITTLE and CUSTER (1963).

1.2. Methods

1.2.1. Enzymatic degradation of caseins. Aliquots of 4 ml of whole and $\varkappa$-casein solutions, resp., obtained from fresh, pasteurized and hydrogen peroxide treated milks were transferred into dry test tubes and preheated to 35 °C. After the temperature had been reached, 0.1 ml portions of enzyme solution were added. Three tubes each were removed after 5, 10, 15, 30 and 60 min, resp., the contents mixed with 4 ml of 24% trichloroacetic acid (TCA), then filtered through *Macherey–Nagel* 640 D filter paper. Subsequently, the tubes were rinsed and the cake washed with 2 ml of 12% TCA. The combined filtrates were subjected to NPN determination by the micro-*Kjeldahl* method using 0.01 *N* solutions for titration. Blanks (0 reaction time) containing heat denatured enzyme (15 min, 90 °C) were prepared in a similar way. NPN was expressed as % of total N.

The concentrations and amounts of the caseins as well as the activities and protein contents of the enzyme preparations in the reaction mixture are given in Table 1.

Table 1

Compositions of the reaction mixtures used to follow NPN-formation from whole and $\varkappa$-caseins as induced by crystalline chymosin and a purified microbial coagulant from Endothia parasitica, *resp.*

Substrate	Concentration (%, w/v)	Amount (mg)	Enzyme				Enzyme / Substrate (SU mg^{-1})
			Chymosin		Microbial rennet		
			activity (SU)	protein (μg)	activity (SU)	protein (μg)	
Whole casein	2.63	108	5.33	6.39	5.33	23.8	0.05
$\varkappa$-casein	0.39	16	3.16	3.79	3.16	14.1	0.20

Volume of reaction mixtures: 4.1 ml.
SU = *Soxhlet* unit.

For electrophoretic studies of $\varkappa$-casein degradation, reaction conditions and enzyme : substrate ratios were identical with those applied for NPN-determinations, except that the reaction volume was 1.1 ml instead of 4.1 ml and the substrate concentration was 0.36% (w/v) instead of 0.39% (w/v). After periods of 5, 10, 30, 60 and 1320 min, resp., the reaction was stopped by adding 1 ml of veronal buffer pH 8.6. The solutions were kept at –20 °C until use. Polyacrylamide gel electrophoresis (PAGE) was carried out as described earlier (VÁMOS-VIGYÁZÓ *et al.*, 1978), applying 0.53 mg protein per gel tube.

All experiments were carried out in triplicate. Means and standard deviations of NPN values were calculated and compared by analysis of variance and the t test.

2. Results

2.1. Non-protein nitrogen liberation from whole and $\varkappa$-caseins by chymosin and the microbial coagulant

The time-course of NPN-formation from whole and $\varkappa$-caseins I, II and III during the action of CR and MR is represented in Fig. 1. The statistical evaluation of the data by analysis of variance is summarized in *Tables 2–5.*

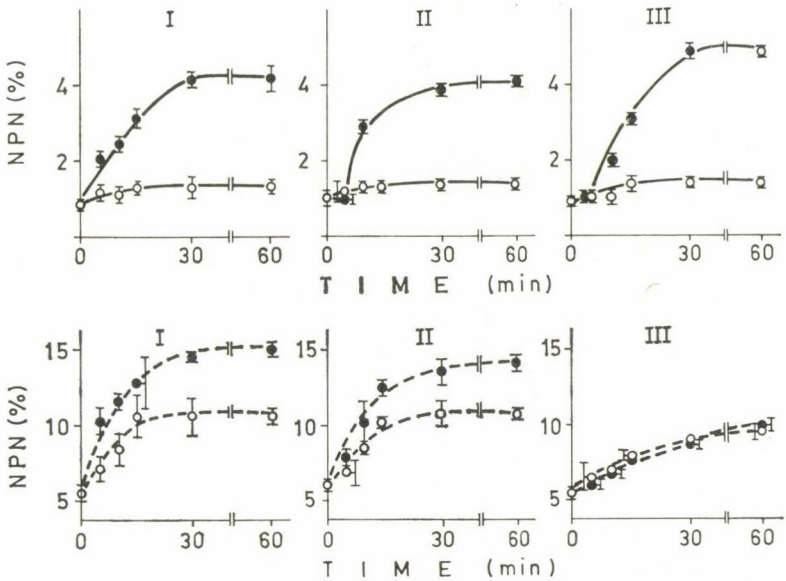

Fig. 1. Non-protein nitrogen (NPN) formation from whole (full line) and $\varkappa$-casein (broken line) prepared from fresh (I), pasteurized (II) and hydrogen peroxide treated (III) cow's milk during the action of chymosin (open circles) and the microbial coagulant (full circles). Experimental conditions: pH 6,0, 35 °C; composition of the reaction mixture as in Table 1. NPN is expressed as % of total N. The vertical bars represent standard deviations. $n = 3$

Table 2

Comparison of differences in mean NPN formed at 35 °C by chymosin, during various periods, from whole caseins prepared from fresh (I), pasteurized (II) and hydrogen peroxide treated (III) skim-milks
For experimental conditions see Table 1 and Fig. 1

↓ Time (min) →	0		5			10			15			30			60		
	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
(I)	*0.17*	0.03	*0.27*	*0.37*	*0.20*	*0.27*	*0.47*	*0.17*	*0.40*	*0.47*	*0.17*	*0.47*	*0.52*	*0.57*	*0.41*	*0.54*	*0.57*
0 (II)		*0.20*	*0.10*	*0.20*	*0.03*	*0.10*	*0.30*	0.00	*0.23*	*0.30*	*0.27*	*0.30*	*0.35*	*0.37*	*0.24*	*0.37*	*0.40*
(III)			*0.30*	*0.40*	*0.23*	*0.30*	*0.50*	*0.20*	*0.43*	*0.50*	*0.20*	*0.50*	*0.55*	*0.60*	*0.44*	*0.57*	*0.60*
(I)				*0.10*	0.07	0.00	*0.20*	*0.10*	*0.17*	*0.20*	*0.30*	*0.20*	*0.25*	*0.30*	*0.14*	*0.27*	*0.30*
5 (II)					*0.17*	*0.10*	*0.10*	*0.20*	0.07	*0.10*	*0.20*	*0.10*	*0.15*	*0.20*	0.04	*0.17*	*0.20*
(III)						0.07	*0.27*	0.07	*0.37*	*0.27*	*0.37*	*0.27*	*0.32*	*0.37*	*0.21*	*0.66*	*0.37*
(I)							*0.20*	*0.10*	*0.13*	*0.20*	*0.30*	*0.20*	*0.25*	*0.30*	*0.14*	*0.27*	*0.30*
10 (II)								*0.30*	0.03	0.00	*0.10*	0.00	0.05	*0.10*	0.06	0.07	*0.10*
(III)									*0.23*	*0.30*	*0.40*	*0.30*	*0.35*	*0.40*	*0.24*	*0.37*	*0.40*
(I)										0.07	*0.17*	0.07	*0.12*	*0.17*	0.01	*0.14*	*0.17*
15 (II)											*0.10*	0.00	0.05	*0.10*	0.06	0.07	*0.10*
(III)												*0.10*	0.05	0.00	*0.16*	0.03	0.00
(I)													0.05	*0.10*	0.06	*0.11*	*0.16*
30 (II)														0.05	*0.11*	0.02	0.05
(III)															*0.16*	0.03	0.00
60 (I)																*0.13*	*0.16*
(II)																	0.03

Least significant difference (P = 95%): 0.08%.
(Differences are expressed in % NPN as related to total N. Digits in italics indicate significant differences.)

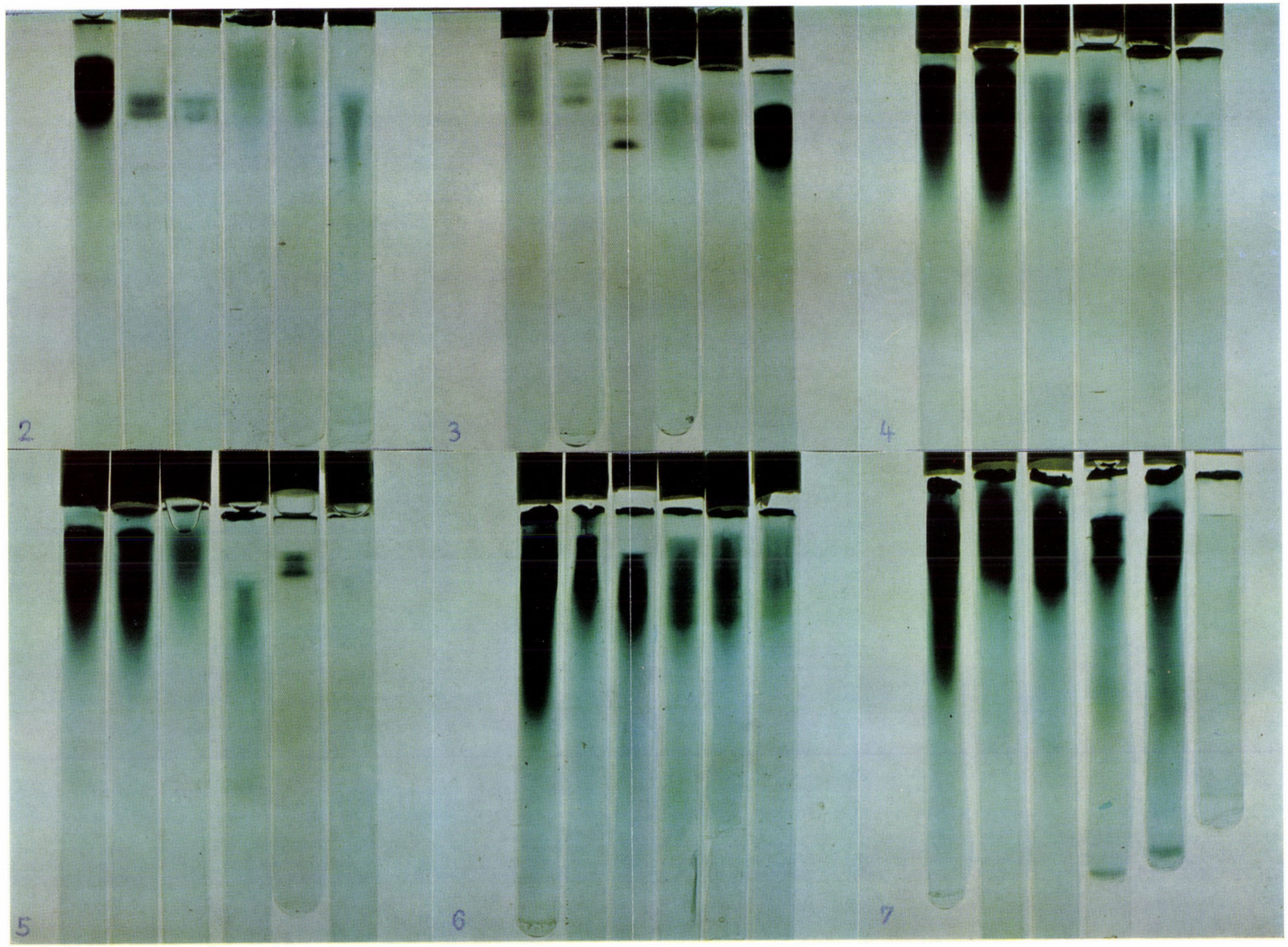

Fig. 2. Electrophoretic patterns of κ-casein prepared from fresh skim-milk, after incubation with chymosin. Incubation time in min and number of protein fractions (in brackets), from left to right: 0 (1), 5 (2), 15 (2), 30 (1), 60 (2), 1320 (3). Composition of the reaction mixture as in *Table 1*. Conditions of the reaction: pH 6.0, 35 °C. Conditions of electrophoresis: 5% polyacrylamide gel, pH 8.6 veronal buffer; electrophoresis at 15 °C, 5 mA per gel tube, 45 min; staining with Amido Black

Fig. 3. Electrophoretic patterns of κ-casein prepared from fresh skim-milk, after incubation with purified *Endothia parasitica* rennet. Incubation time in min and number of protein fractions (in brackets), from right to left: 0 (1), 5 (2), 15 (3), 30 (3), 60 (3), 1320 (3). For experimental conditions see legend to *Fig. 2*

Fig. 4. Electrophoretic patterns of κ-casein prepared from pasteurized skim-milk, after incubation with chymosin. Incubation time in min and number of protein fractions (in brackets), from left to right: 0 (4), 5 (2), 15 (1), 30 (2), 60 (3), 1320 (5). For experimental conditions see legend to *Fig. 2*

Fig. 5. Electrophoretic patterns of κ-casein prepared from pasteurized skim-milk, after incubation with purified *Endothia parasitica* rennet. Incubation time in min and number of protein fractions (in brackets), from left to right: 0 (4), 5 (3), 15 (1), 30 (3), 60 (3), 1320 (1). For experimental conditions see legend to *Fig. 2*

Fig. 6. Electrophoretic patterns of κ-casein prepared from hydrogen peroxide treated skim-milk, after incubation with chymosin. Incubation time in min and number of protein fractions (in brackets), from left to right: 0 (1), 5 (1), 15 (1), 30 (1), 60 (3), 1320 (3). For experimental conditions see legend to *Fig. 2*

Fig. 7. Electrophoretic patterns of κ-casein prepared from hydrogen peroxide treated skim-milk, after incubation with purified *Endothia parasitica* rennet. Incubation time in min and number of protein fractions (in brackets), from left to right: 0 (1), 5 (2), 15 (1), 30 (2), 60 (2), 1320 (2). For experimental conditions see legend to *Fig. 2*

Table 3

Comparison of differences in mean NPN formed at 35 °C by the microbial rennet, during various periods, from whole caseins prepared from fresh (I), pasteurized (II) and hydrogen peroxide treated skim-milks

For experimental conditions see Table 1 and Fig. 1

Time (min) → / ↓	0		5			10			15			30			60		
	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
(I)	*0.10*	0.00	*1.17*	*0.10*	*0.10*	*1.54*	—	*1.10*	*2.24*	*2.00*	*2.14*	*3.30*	*3.00*	*3.97*	*3.27*	*3.20*	*4.00*
0 (II)		*0.10*	*1.07*	0.00	0.00	*1.44*	—	*1.00*	*1.93*	*1.90*	*2.04*	*3.20*	*2.90*	*3.87*	*3.17*	*3.10*	*3.90*
(III)			*1.17*	*0.10*	*0.10*	*1.54*	—	*1.10*	*2.24*	*2.00*	*2.14*	*3.30*	*3.00*	*3.97*	*3.27*	*3.20*	*4.00*
(I)				*1.07*	*1.07*	*0.37*	—	*0.17*	*1.07*	*0.83*	*0.96*	*2.13*	*1.83*	*2.80*	*2.10*	*2.03*	*2.83*
5 (II)					0.00	*1.44*	—	*1.00*	*2.14*	*1.90*	*2.04*	*3.24*	*2.90*	*3.87*	*3.17*	*3.10*	*3.90*
(III)						*1.44*	—	*1.00*	*2.14*	*1.90*	*2.04*	*3.24*	*2.90*	*3.87*	*3.17*	*3.10*	*3.90*
(I)							—	*2.04*	*0.70*	*0.46*	*0.60*	*1.76*	*1.46*	*2.43*	*1.73*	*1.66*	*2.46*
10 (II)								—	—	—	—	—	—	—	—	—	—
(III)									*1.14*	*0.90*	*1.04*	*2.20*	*1.90*	*2.87*	*2.17*	*2.10*	*2.90*
(I)										*0.24*	*0.10*	*1.06*	*0.76*	*1.73*	*1.03*	*0.96*	*1.76*
15 (II)											*0.14*	*1.30*	*1.00*	*1.97*	*1.27*	*1.20*	*2.00*
(III)												*1.16*	*0.86*	*1.83*	*1.13*	*1.06*	*1.86*
(I)													*0.30*	*0.67*	0.03	*0.10*	*0.70*
30 (II)														*0.97*	*0.27*	*0.20*	*1.00*
(III)															*0.70*	*0.77*	0.03
60 (I)																0.07	*0.73*
(II)																	*0.80*

Least significant difference (P = 95%): 0.07%.

— : no measurement

(Differences are expressed in % NPN as related to total N. Digits in italics indicate significant differences.)

Table 4

Comparison of differences in mean NPN formed at 35 °C by chymosin, during various periods, from κ-caseins obtained from fresh (I), pasteurized (II) and hydrogen peroxide treated (III) skim-milks
For experimental conditions see Table 1 and Fig. 1

↓ Time (min) →	0		5			10			15			30			60		
	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
(I)	*0.50*	0.10	*1.57*	*1.70*	*1.00*	*3.10*	*2.90*	*1.50*	*5.10*	*4.70*	*2.57*	*5.10*	*5.30*	*3.55*	*5.10*	*5.10*	*4.40*
0 (II)		*0.40*	*1.07*	*1.20*	*0.50*	*2.60*	*2.40*	*1.05*	*4.60*	*4.20*	*2.07*	*4.60*	*4.80*	*3.05*	*4.60*	*4.20*	*3.90*
(III)			*1.47*	*2.40*	*0.90*	*3.00*	*2.80*	*1.45*	*5.00*	*4.60*	*2.47*	*5.00*	*5.20*	*3.45*	*5.00*	*5.20*	*4.30*
(I)				*0.13*	*0.57*	*1.53*	*1.33*	0.02	*3.53*	*3.13*	*1.00*	*3.53*	*3.73*	*2.83*	*3.53*	*3.73*	*2.83*
5 (II)					*0.70*	*1.40*	*1.20*	*0.90*	*3.40*	*3.00*	*0.87*	*3.40*	*3.60*	*2.70*	*3.40*	*3.60*	*2.40*
(III)						0.02	*0.15*	*0.55*	*4.10*	*3.70*	*1.57*	*4.10*	*4.30*	*3.40*	*4.10*	*4.30*	*3.40*
(I)							*0.20*	*1.55*	*2.00*	*1.60*	*0.53*	*2.00*	*2.20*	*0.45*	*2.00*	*2.20*	*1.30*
10 (II)								*1.35*	*2.20*	*1.80*	*0.33*	*2.20*	*2.40*	*0.65*	*2.20*	*2.40*	*1.50*
(III)									*3.55*	*3.15*	*1.02*	*3.55*	*3.75*	*2.00*	*3.55*	*3.70*	*2.85*
(I)										*0.40*	*2.53*	0.00	*0.20*	*1.55*	0.00	*0.20*	*0.70*
15 (II)											*2.13*	*0.40*	*0.60*	*2.73*	*0.40*	*0.60*	*0.30*
(III)												*2.53*	*2.73*	*0.98*	*2.53*	*2.73*	*1.83*
(I)													*0.20*	*1.55*	0.00	*0.20*	*0.70*
30 (II)														*1.75*	*0.20*	0.00	*0.90*
(III)															*1.55*	*1.75*	*0.85*
(I)																*0.20*	*0.70*
60 (II)																	*0.90*

Least significant difference (P = 95%): 0.12%.
(Differences are expressed in % NPN as related to total N. Digits in italics indicate significant differences.)

Table 5

Comparison of differences in mean NPN formed at 35 °C by the microbial rennet, during various periods, from κ-caseins obtained from fresh (I), pasteurized (II) and hydrogen peroxide treated (III) skim-milks

For experimental conditions see Table 1 and Fig. 1

Time → / ↓ (min)		0		5			10			15			30			60		
		II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
0	(I)	*0.50*	0.00	*4.90*	*1.70*	*0.90*	*5.90*	*4.60*	*1.40*	*7.20*	*6.90*	*2.25*	*9.60*	*8.60*	*3.30*	*9.30*	*8.30*	*4.40*
	(II)		*0.50*	*4.40*	*1.20*	*0.50*	*5.40*	*4.10*	*0.90*	*6.70*	*6.40*	*1.75*	*8.50*	*7.50*	*2.80*	*8.80*	*7.80*	*3.90*
	(III)			*4.90*	*1.70*	*0.90*	*5.90*	*4.60*	*1.40*	*7.20*	*6.90*	*2.25*	*9.60*	*8.60*	*3.30*	*9.30*	*8.30*	*4.40*
5	(I)				*2.50*	*4.50*	*1.00*	*0.30*	*3.50*	*2.30*	*2.00*	*2.65*	*4.10*	*3.10*	*1.60*	*4.40*	*3.40*	*0.50*
	(II)					*2.00*	*3.50*	*2.20*	*1.00*	*4.80*	*4.50*	0.15	*6.60*	*5.60*	*0.90*	*6.90*	*5.90*	*2.00*
	(III)						*5.50*	*4.20*	*1.00*	*6.80*	*6.50*	*1.85*	*8.60*	*7.60*	*2.90*	*8.90*	*7.90*	*4.00*
10	(I)							*1.30*	*4.50*	*1.30*	*1.00*	*3.65*	*3.10*	*2.10*	*2.45*	*3.40*	*2.40*	*1.50*
	(II)								*3.20*	*2.60*	*2.30*	*2.35*	*4.40*	*3.40*	*1.30*	*4.70*	*3.70*	*0.20*
	(III)									*5.80*	*5.50*	*0.85*	*7.60*	*6.60*	*1.90*	*7.90*	*6.90*	*3.00*
15	(I)										*0.30*	*4.95*	*1.80*	*0.80*	*3.90*	*2.10*	*1.10*	*2.80*
	(II)											*4.65*	*2.10*	*1.10*	*3.60*	*2.40*	*1.40*	*2.50*
	(III)												*6.75*	*5.75*	*1.05*	*7.05*	*6.05*	*2.15*
30	(I)													*1.00*	*5.70*	*0.30*	*0.70*	*4.60*
	(II)														*0.90*	*1.30*	*0.30*	*3.60*
	(III)															*6.00*	*5.00*	*1.10*
60	(I)																*1.00*	*4.90*
	(II)																	*3.90*

Least significant difference (P = 95%): 0.17%.

(Differences are expressed in % NPN as related to total N. Digits in italics indicate significant differences.)

Total NPN formed from whole caseins I, II and III during 1 h of incubation with CR was 0.41, 0.37 and 0.6%, resp. The corresponding values obtained for MR were about tenfold: 3.2, 3.13 and 3.93%, resp. The initial value of NPN was higher for casein II (however, in the case of CR, the values obtained for caseins I and II did not differ significantly). With CR, the reaction came to a standstill after 10 to 15 min, while with MR NPN-formation increased up to 30 min, in the case of casein II even up to 60 min, the end of the observation period. With CR, NPN-formation from all three caseins began right after the addition of the enzyme, while with MR a 5-min lag-period could be observed on substrates II and III.

Results obtained with $\varkappa$-caseins I and II were essentially similar in trend to those obtained with whole casein: NPN formed by CR during 1 h of incubation amounted to 5.1 and 4.8%, resp., while MR released 8.3 and 7.8%. The initial value of NPN was significantly higher for $\varkappa$-casein II. The onset of the reaction was instantaneous with both enzymes on both substrates. With CR, liberation of NPN from $\varkappa$-caseins I and II continued up to 15 and 30 min, resp. With MR, the reaction was found to proceed throughout the observation period. Findings obtained with $\varkappa$-casein III were strikingly different in every respect. The final amounts of NPN set free by the two enzymes were practically identical: 4.3 and 4.4% (the difference was not significant: $t = 1.02$, $df = 4$). So were the values obtained after identical periods of incubation. The time course of the reaction with this substrate was not similar to that observed with the previous ones. The lag-phase observed in NPN release from whole casein III was not detectable in this case.

2.2. Changes brought about by the two enzymes in the electrophoretic behaviour of $\varkappa$-caseins

As no dissociating agents have been applied in the $\varkappa$-casein solutions subjected to electrophoresis, the zones obtained were not too distinct. Nevertheless, the marked differences obtained for the $\varkappa$-caseins from milks treated in different ways as well as for their degradation by the two enzymes permit a qualitative evaluation of the results. Photographs of the original electrophoretograms obtained after different periods of incubation are shown in Figs. 2–7.

As can be seen, all $\varkappa$-caseins show a slight contamination by β-casein which appears to be strongest in preparation II. Apart from this, $\varkappa$-caseins I and III both consist of a single zone, however, of different appearance. $\varkappa$-casein II contains, beside the main fraction and the β-casein contamination, two faint bands of low mobilities.

The breakdown of $\varkappa$-casein I seems to be achieved by both enzymes within the first 5 min of the reaction: two distinct bands each appear instead of the dark zone of the original compounds (Figs. 2 and 3). During the following period strong deviations can be observed in the actions of the two

enzymes. With CR, the two bands formed during the first 5 min persist up to 30 min, when a new phase of transformation seems to begin. This results, after 22 h, in a diffuse and two sharp protein zones, the latter of very high mobilities. With MR, breakdown gives rise, after 15 min, to a third sharp band. This pattern persists up to 30 min. The 60-min sample still shows 3 protein zones, however of reduced mobility. The final pattern contains two or three diffuse protein fractions of low and – in contrast to $\varkappa$-casein I treated with CR – but one band of high mobility.

Summarizing the results obtained with $\varkappa$-casein I it can be said that para-$\varkappa$-casein formation takes place at the same rate with both coagulants. Subsequent non-specific proteolysis starts somewhat earlier with MR and yields different products with the two enzymes.

With $\varkappa$-caseins II detailed interpretation of the results is difficult, owing to the initial heterogeneity of the substrate (Figs. 4 and 5). The beginning of the reaction seems to be delayed with both enzymes up to 15 min. From 30 min onward the divergence of protein patterns as obtained with the two enzymes progresses with reaction time. After 60 min, two low-mobility bands are characteristic of the sample treated with MR and an elongated zone with diffuse edges of the one digested with CR. These patterns are somewhat similar to those obtained with $\varkappa$-casein I after identical periods. With CR, further changes up to 22 h consist mainly in the formation of two additional bands of very high mobility. This pattern, too, reminds of the respective result obtained with $\varkappa$-casein I, whereas in the case of MR, protein hydrolysis was nearly complete in 22 h.

Para-$\varkappa$-casein formation from $\varkappa$-casein III was protracted with both enzymes as compared to $\varkappa$-casein I. With MR, part of the original protein seemed to persist up to 60 min, while this was not the case with CR. With this latter enzyme two of the characteristic low-mobility protein zones first appearing in the 30-min sample were still present after 22 h, while with MR, nearly all proteins were hydrolyzed by this time to low molecular weight compounds. A high mobility fraction with distinct edges that first appeared in the sample obtained after 30 min of incubation with MR and was still discernible after 22 h, did not appear at all in the patterns obtained with CR.

3. Conclusions

3.1. Non-protein nitrogen release from whole and $\varkappa$-caseins upon enzyme action

Both substrates were found to have measurable NPN-contents prior to the exposure to enzyme action (*i.e.* when mixed with inactivated enzyme). The values ranged from 0.8 to 1.0% and from 5.5 to 6.0% of total N for whole and for $\varkappa$-casein, resp. Their ratio suggests that also in whole casein the $\varkappa$-com-

ponent might account for the presence of NPN. This phenomenon as observed by several authors has been attributed to acid precipitation or urea treatment used in preparing $\varkappa$-casein and resulting in a degradation similar to that brought about by the action of chymosin (BEEBY & NITSCHMANN, 1963). The values of NPN as established in the present study are in agreement with the findings of others (EL-NEGOUMY, 1968; REPS *et al.*, 1970; ITOH & THOMASOW, 1971; ITOH, 1972; VANDERPOORTEN & WECKX, 1972).

The release of NPN as it occurs upon the action of chymosin or microbial coagulants has been extensively studied by many authors using whole casein and casein fractions obtained from fresh milk. Substrates prepared from milk treated by heat or hydrogen peroxide have been investigated to a lesser extent.

The stronger NPN formation from whole caseins by MR as compared to CR is in complete agreement with the results of the electrophoretic study cited earlier (VÁMOS-VIGYÁZÓ *et al.*, 1980) as well as with the findings of VANDERPOORTEN & WECKX (1972). However, on $\varkappa$-casein from fresh milk, the latter authors established a similar time course of NPN release for both enzymes, whereby the values were always somewhat lower with the *E. parasitica* enzyme than with veal rennet. This might be due to impurities of the latter preparation as used by these authors and is contrary not only to the results of the present study, but also to the data published by PAQUET & ALAIS (1978), according to which more degradation products soluble in 12% TCA were obtained from $\varkappa$-casein with *E. parasitica* rennet than with veal rennet. The number of the peptide bonds of $\varkappa$-casein hydrolyzed (at pH 3.0) under identical periods was equally higher with the *Endothia parasitica* enzyme (TAM & WHITAKER, 1972).

In agreement with the data presented here, proteolysis of casein was found also by others to be significantly influenced by heat treatment of casein and whey solutions in the presence of calcium ions: less NPN was formed and the rate of the process was lowered (DAMICZ *et al.*, 1975). Comparing the actions of rennin, pepsin and pronase, the same authors came to the conclusion that the effect of $\varkappa$-casein-β-lactoglobulin complex formation on casein hydrolysis depended on the enzyme used.

No author seems to have observed the 5-min lag phase of NPN formation as found in this study with MR and whole caseins II and III. This phenomenon was not found to occur with $\varkappa$-casein, probably owing to the higher enzyme : substrate ratio applied. The somewhat slighter extent of NPN release as brought about by MR on substrate II supports the electrophoretic results (VÁMOS-VIGYÁZÓ *et al.*, 1980) and indicates that heat-induced complex formation of $\varkappa$-casein exerts a slight inhibitory effect on the proteolytic activity of this enzyme. Hydrogen peroxide treatment seems to inhibit $\varkappa$-casein proteolysis by both enzymes to a certain extent. The curve obtained for the hydrolysis

of whole casein III by MR suggests that the inhibitory effect of hydrogen peroxide treatment on $\varkappa$-casein degradation is largely compensated by enhanced proteolysis of the other casein constituents.

3.2. Changes in the electrophoretic behaviour of $\varkappa$-casein

As mentioned before, electrophoresis of $\varkappa$-casein under the conditions applied did not yield high resolution, owing to the absence of dissociating agents from the system. The same had been reported earlier by WOYCHIK (1965). However, in view of the partial degradation of $\varkappa$-casein to para-$\varkappa$-casein by urea (BEEBY & NITSCHMANN, 1963) as well as the reduction of S–S bonds by mercapto ethanol (WOYCHIK, 1965), it was thought safer to preserve the native structure of $\varkappa$-casein as far as possible, in order to be able to study the respective actions of the two enzymes under conditions similar to those prevailing in milk. In spite of this precaution some degradation of $\varkappa$-casein during preparation could not be prevented. This manifested itself in the release of NPN as mentioned in para 3.1. Moreover, the precipitate on top of the gels (Figs. 2–7) giving the characteristic protein stain with Amido Black, might be due to para-$\varkappa$-casein, as this compound is positively charged and insoluble in basic systems (EL-NEGOUMY, 1968; VANDERPOORTEN & WECKX, 1972). Changes in electrophoretic patterns have nevertheless been considered to give a better evaluation of proteolysis as induced by rennet extract than NPN formation, since the pH optimum of the latter reaction is much lower than that applied in cheese-making, *i.e.* milk clotting (FOX, 1969).

Under the experimental conditions chosen in the present study, the transformation of $\varkappa$-casein I by either enzyme seemed to be accomplished within the first 5 min of the reaction as marked by the appearance of two distinct bands in the electrophoretograms instead of the zone originally present. A similar observation was made by ITOH and THOMASOW (1971) as well as by ITOH (1972) using rennet and other milk clotting enzymes. PAQUET and ALAIS (1978) reported the two fractions appearing at the migration distance corresponding approximately to that of $\varkappa$-casein to persist up to 90 min, independently of the enzyme applied. Figs. 2 and 3 show a quicker transformation of these patterns and differences, in this respect, between CR and MR. The same authors have pointed out that, as $\varkappa$-casein transformation to para-$\varkappa$-casein is complete in 10 min and this latter protein migrates to the cathode, the two fractions must be degradation products of other casein components. This again does not apply to the results obtained with practically pure $\varkappa$-casein as presented here. On the other hand, using high resolution starch gel electrophoresis in alkaline medium (pH 8.60), after 60 min of incubation with crystalline rennin, EL-NEGOUMY (1968) detected at least 11 breakdown products of $\varkappa$-casein, the majority of which were *negatively* charged.

Moreover, according to the same author, $\varkappa$-casein was the only component of whole casein attacked by rennin within 50 min and yielding several slow and fast moving components. Thus, the various fast moving breakdown products of casein as attributed earlier (VÁMOS-VIGYÁZÓ *et al.*, 1980) to decomposition of α_s- and β-casein might equally be due to $\varkappa$-casein degradation.

As can be seen, the results obtained by various authors as well as their interpretation disagree largely, probably owing, at least partly, to differences in experimental conditions.

Further changes in the electrophoretic patterns of $\varkappa$-casein I revealed differences between the actions of the two enzymes, especially after prolonged incubation. The differences were more pronounced with $\varkappa$-caseins II and III and were in agreement with observations in cheese-making practice (apparent reduction of clotting power of both enzymes in pasteurized milk and prolongation of the coagulation process with MR in hydrogen peroxide treated milk) as well as with the results obtained on whole casein substrates (VÁMOS-VIGYÁZÓ *et al.*, 1980).

The differences in the action of chymosin and the enzyme from *Endothia parasitica* were also demonstrated by KOVÁCS-PROSZT and SANNER (1973) who investigated the macropeptides released by the two coagulants from acid casein and $\varkappa$-casein.

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Abstracts

of papers presented at the

COLLOQUIUM ON ENZYMATIC ANALYSIS AND ENZYME DIAGNOSTICS

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The *Biochemical Section of the Hungarian Chemical Society*, the *Microbiological Section* of the *Hungarian Scientific Society for Food Industry* and the *Hungarian Society of Clinical Pathology* jointly sponsored a colloquium mainly dealing with the biochemical and food science aspects of the subject matter indicated in the title.

Several of the 58 papers submitted are of interest to the food scientist and their abstracts are presented below.

ROLE AND FUTURE OF ENZYMATIC METHODS IN MODERN FOOD ANALYSIS

I. INTRODUCTION, THEORY AND ANALYSIS OF FOODS OF ANIMAL ORIGIN

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The development of enzymology was connected with that of food technology. Even procedures of enzymatic analysis were used in the past century to check the right execution of heat treatment. The enzymatic analysis of foods has been developed to a large extent in the 20th century as well. The usefulness of enzymatic analysis is attributed to the following characteristics of the enzymes: (a) as the result of their specificity enzymes may be used to determine compounds the separation of which is extremely difficult or impossible by other means; (b) enzymes are very sensitive to environmental conditions and are of use in the investigation of inhibiting or activating factors. In enzymatic analysis the laws of enzyme kinetics must always be taken into consideration. By measurement of reaction rates, *Michaelis* constants of several one-substrate or multi-substrate reactions may be determined. Enzymatic analysis is suitable for the determination of enzyme activities or metabolite concentrations, for determinations by means of coupled reactions, for the investigation of the types of inhibition, *etc.*

Enzymatic analysis is mainly used in foods to determine endogenous food enzymes, to check the activity of the enzyme preparations used in food processing and to determine the amounts of food components and food additives.

1. Meat. The detection of *glutamate oxaloacetate* transaminase isoenzymes may be of use to distinguish fresh and frozen pork or beef, as the isoenzyme of the mitochondria finds access to the sarcoplasm through the membranes injured by the freezing process. In order to check the efficiency of heat treatment of meat and meat products, the determination of the rate of inactivation of muscle phosphatase or of carboxyl esterase is applicable. The activity of the proteolytic enzymes used in meat tenderizing may be easily checked by determination of the amount of tyrosine released from a well-defined protein, *e.g.* Bacto-haemoglobin, upon enzyme action. Methods measuring protease activity are also recommended to compare different enzyme preparations and to determine losses of activity during storage.

Lactic acid, the end product of anaerobic glycolysis may easily be determined by means of the reaction lactate $\rightarrow$ pyruvate, using lactate dehydrogenase in alkaline solution and shifting the equilibrium with hydrazine to complete the transformation which may be followed by the change in absorbance at 340 nm.

To determine *ethanol* content in meat products, ethanol separated selectively by steam distillation is converted to acetaldehyde by alcohol dehydrogenase. The hydrogen liberated in the reaction reduces NAD^+ as in the reaction above. This transformation, too, may be followed spectrophotometrically at 340 nm.

Monosodium glutamate, the flavouring substance of meat products may be determined by two methods. Either CO_2 is released from glutamic acid by action of glutamate decarboxylase and determined in a Warburg apparatus, or glutamate is oxidized to α-keto-glutarate by glutamate dehydrogenase and the NAD^+–NADH transformation followed spectrophotometrically at 340 nm. The transformation is quantitative in alkaline medium, in the presence of hydrazine and of excess NAD^+.

Sucrose in sausages may be determined by the following specific reactions: sucrose is at first converted to glucose and fructose, these compounds are converted by hexokinase to the corresponding phosphates and glucose-6-phosphate is oxidized, by glucose-6-phosphate dehydrogenase, to 6-phosphogluconate. ATP is the phosphate donor, NADP the hydrogen acceptor. The amount of NADPH formed is determined spectrophotometrically at 340 nm.

The addition of *corn starch syrup* may be determined by the copper reduction method, after hydrolysis with α- and β-amylase. The hydrolysis of starch to glucose by other enzymes is also feasible and glucose may be determined by the methods mentioned. The addition of skim-milk may be deter-

mined on the basis of its lactose content. After splitting lactose to monosaccharides by β-galactosidase, the glucose content may be determined by the hexokinase method mentioned above. In meat products containing starch syrup and dried skim-milk, sucrose has to be destroyed by yeasts, other sugars by maltase and glucose oxidase, prior to the determination of lactose.

For the detection of *sodium caseinate* in meat products, an agar gel precipitation method has been developed on the principle that some proteases, *e.g.* chymotrypsin, precipitate sodium caseinate, while proteins such as dried skim-milk or soybean proteins do not. Inorganic pyrophosphate additives may be determined by specific reactions: yeast pyrophosphatase releases inorganic pyrophosphate which, in turn is split in the presence of Mg^{2+}, to 2 molecules of orthophosphate, and the latter can be determined spectrophotometrically by colour reactions.

2. Fish. Frozen marine fish products may be distinguished by the different electrophoretic mobilities of their lactate dehydrogenase isoenzymes.

3. Milk and dairy products. Milk contains numerous enzymes, part of which are natural constituents of milk, other enzymes occur as bacterial products or as the result of disturbances of metabolism. In practice, inactivation of alkaline phosphatase in milk and dairy products is used to check whether pasteurization was carried out at the suitable temperature. The inactivation of peroxidase may be used to detect heat treatments performed at higher temperatures. The growth of bacteria of enhanced reductase activity may be detected by means of redox indicators.

Lactic acid and pyruvic acid, both formed upon the action of lactate dehydrogenase, can be determined spectrophotometrically, even by automated method. The use of stereospecific lactate dehydrogenases permits of determining separately L(+)- and D(−)-lactic acid contents.

Several enzyme preparations are used in dairy technology: catalase, rennet, lipase, β-galactosidase, proteases, *etc.* The activity of the individual preparations may be determined by methods described in *Bergmeyer*'s work.

In the determination process of lactose, the disaccharide is hydrolyzed by β-galactosidase to glucose and galactose and either glucose is determined by the hexokinase method or galactose by means of galactose dehydrogenase. In the latter case hydrolysis and dehydrogenation are carried out simultaneously and the amount of NADH formed is determined spectrophotometrically.

The fat content of milk may be determined by the enzymatic assay of glycerol, following extraction and saponification. Phosphorylation of glycerol leads to ADP formation, the latter, in turn, is converted to ATP in the presence of phospho-enol-pyruvate, and the amount of pyruvate is determined by the lactate dehydrogenase method.

4. Eggs. The activities of several enzymes in eggs have been investigated The activities of alkaline phosphatase, glutamate oxaloacetate transaminase and phospholipase were determined.

II. ANALYSIS OF FOODS OF VEGETABLE ORIGIN

This part of the lecture deals with the enzymatic methods used in the analysis of vegetable foods. The assay methods of endogenous enzymes and industrial enzyme preparations involved in processing are included as well.

1. Vegetable oils. Glyceride hydrolysis in oils during frying can be determined by enzymatic analysis of mono- and diglycerides. The assay is based on glycerol determination. Lipoxygenase was applied to determine linoleic acid content in oils and fats of both vegetable and animal origin.

2. Cereals. Amylase activity determinations can easily be carried out using chromogenic starch or amylose substrates. An automated method has been devised in Hungary for measuring protease activity in wheat. Recent papers deal with the assays of peroxidase and lipoxygenase in cereals. The activity of α-amylase enzyme preparations as used in bread-making is most often determined according to the SKB method. The assay of the starch content of flour involves acid hydrolysis and enzymatic glucose determination (glucose oxidase–peroxidase). Damaged starch in flour can be determined by digestion with α-amylase. Dietary fiber content of cereals is assessed from the insoluble residue obtained after digestion with different amylolytic and proteolytic enzymes. L-lysine-decarboxylase lends itself to automated measurement of L-lysine in grain proteins. Sucrose added to bakery products can be determined, after hydrolysis with invertase, on the basis of glucose content (glucose oxidase). Enzymatic methods permit of the simultaneous measurement of sorbitol, sucrose, glucose, fructose and starch content in bakery products.

3. Starch and starch products. Starch yields from different raw materials can be predicted after heat treatment (max. 130 °C) and subsequent hydrolysis to glucose, determining the latter product enzymatically (glucose oxidase or hexokinase). Various automatic assay procedures have been developed to assess the activities of the main enzyme preparations used in starch processing (α-amylase, glucoamylase, glucose isomerase). Glucose content in starch hydrolysates can be determined by the glucose oxidase method using a TRIS-buffer to inhibit α-glucosidase impurities of the enzyme preparation. In the presence of arsenate, maltose phosphorylase offers a specific assay for maltose.

4. Natural sweeteners (sugar, honey, corn syrup) and their raw materials. Peroxidase activity is assessed in sugar beet to predict sugar yields.

The activities of α-amylase, invertase and acid phosphatase in honey are used to detect quality damage caused by too strong heating.

The activity of the invertase preparations added to *fondant* cremes to prevent crystallization is checked on sucrose substrate by the rate of glucose formation (glucose oxidase or hexokinase).

The components of a sugar mixture can be determined simultaneously in an automatic analyzer using several enzymes immobilized on the same support: invertase and glucose oxidase for the determination of sucrose, β-galactosidase + glucose oxidase for that of lactose, and glucoamylase + glucose oxidase for the determination of maltose. According to another automated procedure sucrose, glucose and fructose are determined with a series of enzymes covalently bound to cyanogen bromide-activated agarose : invertase to hydrolyze sucrose, hexokinase + ATP to phosphorylate monosaccharides, phosphohexose isomerase to convert fructose-6-phosphate into glucose-6-phosphate and glucose-6-phosphate dehydrogenase to transform glucose-6-phosphate into gluconic acid. The reaction is monitored spectrophotometrically (reduction of NADP in the last step). Raffinose can be assessed in various intermediates and by-products of beet sugar manufacture using α-galactosidase to split off galactose which is then treated with galactose dehydrogenase (see Part I).

5. Fermented beverages (wine, beer). Among the numerous enzymes present in barley and malt, α-amylase has been tested most often, both qualitatively (electrophoretic isoenzyme patterns) and quantitatively *(Hagberg* falling number). From the grape enzymes, mainly polyphenol oxidase has been investigated.

In beer manufacture utilizing surrogates, complex enzyme preparations of amylolytic, proteolytic, cellulolytic and β-glucan decomposing activities are applied. A special activity assay is based on the measurement of reducing sugars set free by the enzyme from malt β-glucan. The activities of proteolytic enzyme preparations as used for chill-proofing of beer are determined under standardized conditions on Bacto-haemoglobin or casein as substrates.

Acetic acid in beer is assessed with acetate kinase, the isomeric lactic acids with lactate dehydrogenases specific to the stereoisomers. Maltose, sucrose, glucose and fructose in beers of low alcohol content are measured, after enzymatic conversion to glucose, by the hexokinase method. Ethanol and several polyhydric alcohols of wine have been determined enzymatically. The routine assay of ethanol applies alcohol dehydrogenase; acetoin or butane-2,3-diol are assessed with butane-2,3-diol-dehydrogenase. Glycerol is transformed, by glycerol kinase, to glycerol-1-phosphate which, in turn, is converted by glycerol phosphate dehydrogenase to dihydroxy-acetone-phosphate, under simultaneous reduction of NAD^+, the reduced form of which is determined spectrophotometrically. A special assay of acetic acid in red wines and coloured fruit juices involves the action of 3 enzymes (acetate kinase, pyruvate kinase and lactate dehydrogenase). Of the non-volatile acids of wine malic, lactic, citric, succinic, ketoglutaric and gluconic acids have been determined using

different enzymes or coupled enzyme reactions. Glucose + fructose content in wine can be assessed by the hexokinase method.

6. Fruits, vegetables and their products. Polyphenol oxidase, peroxidase, polygalacturonase and pectin methyl esterase are the endogenous enzymes most often studied in fruits and vegetables. Some problems arising during activity measurements such as substrate and product inhibition, different response of isoenzymes to various substrates and consequent non-linearities of kinetic plots have been pointed out by the authors. Methods of measuring the activities of several types of pectolytic enzymes are mentioned, *e.g.* those based on decrease in pectin viscosity (polygalacturonases), titration with NaOH of carboxyl groups set free by pectin methyl esterase, analysis of the end groups split off by the exo-types of polygalacturonases, weight loss of vegetable tissues (macerases) or the apple juice clarifying action. Lyase-type pectolytic enzyme activity is determined spectrophotometrically.

Ascorbic acid oxidase has been used to measure ascorbic acid and dehydro ascorbic acid contents in raw and canned spinach. Iso-citrate dehydrogenase lends itself to the determination of iso-citric acid in citrus juices and is used as a means of detecting falsifications. Ethanol content of fruit juices and jams can be determined with alcohol dehydrogenase, and the artificial sweeteners sorbitol and xylitol with sorbitol dehydrogenase. Sugars occurring in jams and fruit juices (maltose, sucrose, lactose and malto-oligosaccharides) can be assessed, after enzymatic conversion to glucose, with the glucose oxidase – catalase system. Skim-milk content of spray-dried potato puree is determined by its lactose content using β-galactosidase.

The above examples were given to show the increasing importance and possibilities of application of enzymatic methods in food analysis. Widespread introduction of immobilized enzymes and enzyme electrodes which can be re-used several thousand times without deterioration might largely contribute to general adoption of enzymatic substrate analysis in Hungary.

AUTOMATED ENZYMATIC METHODS IN FOOD ANALYSIS

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Analyses may be performed, on the basis of a selected enzyme reaction, by a manual batch method or by an automated one. The latter case might be executed by the mechanization of the batch method or the continuous execution of the reaction. In the present review examples on the application of the continuous analyzer system *Technicon* in food analysis are given.

The models selected represent nearly the entire field of enzymatic analysis. Enzymatic methods can, namely, be divided fundamentally into the following 3 groups:

1. Methods based on substrate transformation or product formation,
2. Methods based on coenzyme changes or function,
3. Methods based on specific inhibition of the enzyme reaction.

In the first two cases the substrate has to be entirely transformed to product in the continuous system, while in the third case the decrease in initial reaction rate as caused by inhibition is measured, *i.e.* only a negligible part of the substrate is transformed to product.

When determining dissolved components, total transformation of the substrate may be performed, also continuously, by the following three systems:

a) admixture of an enzyme dissolved in water and application of a thermostated spiral tube the length of which corresponds to the conversion time of the dissolved substrate,
b) leading the substrate through a micro-column filled with immobilized enzyme,
c) using an enzyme immobilized to the inner surface of an analytical tubular reactor.

Determination of a sample not entirely soluble or a component not easily soluble may be achieved only after separate preparative steps or predigestion. A good example for the latter case is given by the determination of the starch content of starch-containing food raw materials and products.

1. Automated analysis of starch-containing substrates

A common feature of the starch granules found in the cells of kernels of vegetable origin is the protein structure formed by dehydration occurring during ripening. Structure as well as firmness of this protein matrix are characteristic properties of the given cell. In order to dissolve the starch granules, it is necessary to loosen this coat and make it penetrable. In some industrial technologies loosening is achieved by wet heat treatment under pressure (*Henze* procedure in alcohol manufacture), steeping in sulfurous acid (corn starch manufacture) or enzymatic degradation (preparation of wort in brewing). Under laboratory conditions dissolving with cold perchloric acid, hot calcium chloride, *etc.* is applied.

From the above possibilities enzymatic digestion was thought to be applicable for automated starch determination. The working hypothesis of the authors was as follows: in the grist prepared from kernels of vegetable origin, loosening of the protein structure may be carried out by proteolytic enzymes, "liquefaction" or dissolution of the starch granules by alpha-amylase of high temperature optimum and conversion of starch to glucose by the enzyme glucoamylase.

After saccharification the starch content of the samples is present in the form of glucose which may be determined in several ways.

The less expensive and easiest to perform is the ferricyanide procedure, applied also in the author's laboratory for automated starch determination as developed on the basis of the above principles. Details of the method are given in a separate paper.

The ferricyanide method of glucose determination applicable in the concentration range of 0.5 to 5.0 g l^{-1} as used also in the author's laboratory is, however, not always satisfactory. When measuring low enzyme activities or performing studies of enzyme kinetics, a more sensitive method is needed. If this method has to be continuous as well, enzymatic procedures increase the costs to a large extent.

Starting from this consideration, a micro-ferricyanide method has been developed which permits of continuous measurement of reducing sugars in analyzers of the *Technicon* system. The range of measurement of this procedure is, by two orders of magnitude, below that of the method mentioned above, it lends itself to measuring glucose concentrations of 5 to 100 mg l^{-1}. This range of measurement is comparable to that of enzymatic methods, is much less expensive than those, but – as a matter of fact – only specific to reducing sugars.

In kinetic studies on which, in the case of amylolytic enzymes, also a separate paper will report, the reducing action of the substrate itself may have a disturbing effect.

2. Methods based on coenzyme changes or function

Several hundred procedures are known which are based on the analytical application of the coenzymes NAD or NADP as coupled to various dehydrogenases. However, continuous flow-system analyzers are not current in this field, owing mainly to the high prices of the enzymes and coenzymes of the required purity.

Even batch procedures are not cheap. The analysis of glucose-fructose syrup, starting from corn, as performed by the author should be mentioned as an example. For specific measurement of glucose, hexokinase, glucose-6-phosphate-dehydrogenase, ATP and NADP have been used. For fructose measurement hexose-phosphate-isomerase was applied, in addition. Imported chemicals necessary for the determination of the glucose-fructose content of a single sample amount to 40–60 Forints. Although the determination is absolutely specific, flow system continuous analysis is expected to spread only when applying immobilized enzymes of suitable stability. Material costs could be further reduced by a suitable coenzyme regenerating system.

The functions of the coenzymes permit, at the same time, also their determination. Two simple examples should be mentioned: diphenol oxidases

dehydrogenate diphenols only in the presence of Cu^{2+} ions. Glucose isomerase as used in manufacturing glucose-fructose syrup isomerizes only in the presence of Co^{2+} ions. Consequently, the two apoenzymes may be used to determine specifically copper and cobalt ions, resp., on the basis of measuring the action of the regenerated holoenzyme content. By applying dissolved or immobilized enzymes, the methods can easily be transformed into continuous procedures.

3. Utilization of enzyme inhibition for automated analysis

Enzyme inhibition is, in most cases, of similar specificity as enzyme action. The possibilities implied in the topic should be presented through the examples of 3 different types of inhibition:

a) specific competitive inhibition of the enzyme,

b) competitive product inhibition,

c) "reactive" inhibition of enzymes.

Heparin competitively inhibits RN-ase action. The rate of RNA-decomposition as measured at 300 nm is proportional to the heparin content of the sample. The method of the sensitivity of 0.5 to 2.5 mg l^{-1} can be easily automated.

As an example of utilizing product inhibition, the automated phosphate determination as developed by the firm *Corning*, should be mentioned. Alkaline phosphatase has been coupled to porous glass beads. By leading the substrate p-nitrophenyl phosphate through a micro-column filled with the immobilized enzyme, the p-nitrophenol formed is easy to measure. If the substrate solution contains also the inorganic phosphate to be determined, initial reaction rates will be reduced. The inverted values of the inhibited reaction rates vary, in the range of phosphate concentrations between 0.1 and 1 mM l^{-1}, according to a linear function.

The most characteristic example of "reactive" inhibition is cholinesterase inhibition by organophosphates. An account on this procedure can be found in a separate paper (Tóth & László: p. 74 of this volume).

ENZYME ASSAYS BY MEANS OF A CHEMICAL ANALYZING SYSTEM OPERATING ON A NEW PRINCIPLE

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The FP–9 analyzing system (Labsystems OY, Helsinki) consists of a 9-channel photometer, an adjoining desk computer and a thermostating-stirring unit. The photometer measures simultaneously 9 test samples in one s, one

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or several points, thus can be set to kinetic measurements as well. Test substances and reagents can be injected with 9-channel pipettes or feeders, thus several thousand one-point, or several hundred kinetic measurements can be performed per hour. There are practically no methodical limitations, on the basis of the programs developed by the computer manufacturers all photometric measurements, that are known at present can be carried out, while the user himself may also elaborate new programs. Micro methods can be applied, a 300 μl reaction mixture is already well measurable.

The *basic new feature* of the apparatus is that the light beam passes vertically through the cuvettes, and reaches the photocells through the free surface of the liquid. This not only means that the width of the layer depends on the measured sample volume, but it creates, at the same time, entirely new measuring conditions, basically different from that of the known photometers. *What we measure is not concentration, but the absolute quantity of the tested substance*; the measured substance gives equal absorption, irrespective of the quantity of the added, optically inert reagents, *etc.*

Based on the introduced measuring principle and the international definition of enzyme units, we have come to the conclusion that, when enzymes are assayed, the activity, indicated by the NAD–NADH co-enzyme transformation, can be measured with the same program without special calibration, provided the calibration of the apparatus is done according to the molar absorption coefficient of the test sample, *e.g.* NADH.

Our experiments have verified the correctness of the assumption: with the program set on the basis of the "virtual absorption coefficient", applicable to the device, we carried on kinetic lactate-dehydrogenase, creatine-quinase, hydroxy-butirate-dehydrogenase, aspartate- and alanin-aminotransferase assays by applying *Boehringer* UV-test. Our results were checked with General Diagnostics Versatol E and -EN as well as *Boehringer* Precinorm E and Precipath E control serums, which correspond to normal or abnormally increased values measurable in human blood serum. In the other course of the experiments equal serum quantities were measured, but the substrate values varied between 300–600 μl. As expected, we got activity values given for standard serums, irrespective of the measured substratum quantities.

The application of this new measuring system may have extreme advantages in the analytical practice:

- We can simultaneously define different enzymes in identical samples or vice versa, the same enzyme can be defined in different test substances.
- Large test series or smaller series of various tests can be equally quickly measured, what is more, prompt tests can be included any time, and without delay.
- A possible inaccurate measuring of the reagents does not affect the accuracy of the measurement itself.

ENZYME ELECTRODES – POSSIBILITIES AND LIMITS OF THEIR APPLICATION

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Clark, Hicks and *Updike* were the first to report, in 1967, on the preparation of enzyme electrodes. During the relatively short period elapsed since that time the range of selective electroanalytical measuring detectors has been widened on the basis of the works of *Guilbault, Clark, Montalvo, Rechnitz* and many others, by a whole new, dynamically developing group, that of the enzyme electrodes. The importance of enzyme electrodes consists in the fact that, by means of these, a great number of substances can be determined by simple analytical methods which cannot be determined with electrodes of other types. The possibility arises, first of all, to determine substances able to participate in enzyme-catalyzed chemical processes or to influence their rates.

The structure of the enzyme electrodes developed up till now is essentially the same: the enzyme electrodes consist of two parts, *i.e.* of the basic detector and of the reaction layer coating its surface.

From the voltammetric electrodes, the *Clark* oxygen electrode and the platinum and the carbon electrode have been most frequently applied as basic detectors.

In preparing enzyme electrodes, the pH-sensitive glass electrode, the cation selective glass electrode, the ammonia and carbon dioxide gas electrodes are the most frequently used basic detectors operating on a potentiometric principle.

The reaction layer is formed by a thin, generally hydrophilic film, permeable to molecules and ions dissolved in water. The reaction layer contains the enzyme which makes the action of the enzyme electrode possible. During operation or storage of the electrode the catalyst must not leave the reaction layer.

The operation of the electrode is based on a chemical reaction catalyzed by the enzyme. On the measuring surface of the basic electrode coated by the reaction layer and placed into the solution in contact with the electrode, chemical reactions take place in the pesrence of substrate, causing local changes in concentration and ion activity, resp., on the measuring surface of the basic electrode. Material transport as caused by diffusion and aimed at compensating the difference in concentration between the bulk of the solution and the reaction layer, and the chemical reaction taking place in the reaction layer may reach, under suitable conditions, a steady state.

The concentration of the liquid in contact with the measuring surface of the basic electrode definitely depends, all other conditions being equal,

on the rate of the chemical reaction. Consequently, under suitable conditions, the enzyme electrode may show a signal, *i.e.* electrode potential or voltametric current intensity, which depends on the concentration of the substance influencing the rate of the chemical reaction. In this way there is a possibility of measuring the concentrations of the respective substances, substrates, activators, inhibitors or activities of enzymes. Obviously, in enzymatic analysis of a given substance, other substances affecting the rate of the enzyme reaction might present a source of disturbance.

Successful research into the development of enzyme electrodes requires practising of three, relatively distant topics. These are as follows:

- studies connected to enzyme-catalyzed reactions,
- research connected to special application and development of methods of enzyme immobilization and to preparation of reaction layers,
- application and development of electroanalytical detectors.

A great part of the papers published on enzyme electrodes to date report on the conscious, competition-like research work aimed at establishing the properties of the electrodes prepared by means of simple enzyme immobilizing steps from commercial enzyme preparations and yielding changes to be followed with the known basic electrodes on the basis of well-known enzyme reactions.

The electrodes developed as a result of this work as well as their actions were described in detail in the lecture. Further results of importance for analytical practice are to be expected, in the field of research into enzyme electrodes, from co-ordinated work on the three topics mentioned.

The real possibilities of the application of enzyme electrodes are yet difficult to estimate.

Three practical examples pertaining to the application of enzyme electrodes were presented in the lecture, in which the application of enzyme electrodes simplified analytical work otherwise difficult to perform.

– By means of a continuously operating glucose enzyme electrode applied in investigating glucose tolerance, *Sztanyiszláv* and *Arató* successfully eliminated the disturbing consequences of the changes caused by psychic effects that accompany taking of blood.

– *Fritz*, *Nagy*, *Fodor* and *Pungor* applied an urea measuring electrode for quick on-line following of the dissolution process of urea from urea-containing feeds.

– By means of joint application of L- and D-amino acid enzyme electrodes, *Nagy* and *Pungor* developed a simple method to determine the ratio of optical isomers in various amino acid samples.

A SPECIAL MATHEMATICAL METHOD TO EVALUATE ENZYMATIC ANALYSIS

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In the lecture the authors illustrate, on two concrete examples, how grave errors result from establishing the lack of correlation on the basis of the negative result (low correlation coefficient) of linear regression analysis (*e.g.*, no correlation would exist between pressure and volume of ideal gases). Thus, in general, non-linear approximations are necessary to establish the functions describing results of measurements. Applying a method developed by themselves, the authors obtained accurate correlations easy to handle for describing the curve of the radioimmunoassay and the standard curves of conventional GOT and GPT UV-tests according to *Merck*. The essence of the method is, in brief, as follows: a computer program selects, on the basis of error criteria, from a great number of functions the one giving the best approximation of the data of measurement, along with the pertaining constants. By applying the computerized method, the authors developed a procedure which permits to expand the limits of measurement of all kinds of concentration determinations to ranges in which the correlation between concentration and the characteristics measured is non-linear (with most procedures of measurement the concentrations below and above the usual fall into this range).

The programme written in BASIC programme language was run through a computer designed by the authors and presented to the audience.

The computer is a modified SWTP microcomputer. Its memory can be extended to 64 kbyte. Its cycle time is 1 μsec. Eight peripheries according to the standard RS 232 C can be attached directly (*e.g.* a teletypemachine, typewriter, punched-tape reader and puncher, video-terminal, *etc.*). A mini-floppy disk system can be attached as well, with a capacity of maximum 1 Mbyte.

Also a display-terminal belongs to the computer system; this has a screen of 64×16 characters, the cycle time of the screen is 0.8 msec. The rate of transmission can be adjusted between 110 and 2400 Baud when used as terminal. The terminal is equipped with a set of 64 and 128 ASCII characters, resp., and with cursor control characters (erasing of pages, lines or characters, moving of cursor *etc.*). A cassette-interface belongs to the system as well, permitting to attach a common tape recorder to the system as background memory. The capacity of this is 250 kbyte.

The software serving the system is available built in ROM. A 8K BASIC interpreter of relatively high performance, supplied with orders pertaining to handle transcendent functions and string variables, graphics (256 × 140 pixels), has reached completion, an Extended BASIC variant of higher performance to be used with the minifloppy is ready as well.

A particularly great advantage of the system lies in the fact that its production costs are approximately by one order of magnitude below those of the minicomputers.

OBJECTIVE QUALIFICATION OF SUGAR BEETS BY ENZYMATIC ANALYSIS

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Data were found in the literature indicating that correlation exists between peroxidase (POD) activity and quality of sugar beets. Joining into the beet ripening experiments extended over the whole territory of the country by the *Trust of the Sugar Industry*, the authors carried out a fact-finding investigation to establish to which extent POD activity measurements can be used to extend the objective qualification of sugar beets.

The investigations pertain to the year 1977. The average value of final digestion was, on a national level, 15.17%. Homogenized samples were obtained from the ten districts of sugar factories, from 5 locations each, at four dates of the cultivation period, between 11 July and 20 September. POD activity was measured in extracts of roots and leaves by a photometric method, with o-phenylene diamine as substrate.

Changes in POD activity of samples from a given sampling location were investigated as a function of actual sugar content and root weight. In general a negative correlation was found between the parameters studied and POD activity of the roots. POD activity of leaves had a maximum at the end of August. The correlation with the parameters investigated was positive. Similar correlations were obtained for the mean values of the individual districts of sugar factories. The quality of sugar beets is influenced by a number of factors. The type of seed-grain is essential. On the areas investigated, mainly the cultivar β-Monopoli N1 is used, beside this one, the hereditarily single-seeded cultivar β-Poly M102, equally of domestic cultivation, as well as some imported cultivars are applied. The characteristics of the plant are significantly affected by the physical state and character of the soil, too. Sugar beets are most frequently grown on medium hard ground of the type chernozem. It was

studied, whether there was a relationship between POD activity and sugar content of sugar beets of the same cultivar grown in different places of the country on the same type of soil. A correlation was found between the activity of the root and the actual sugar content. A negative correlation was observed between POD activity of beets as measured at an intermediate date and final digestion. Foreign researchers found a relationship between resistance of sugar beets towards infections, infection and POD activity of beets. On the areas investigated moderate contaminations with *Cercospora beticola* pertaining to mycotic diseases occurred. It was investigated, how POD activity of sugar beets of the same cultivar grown on soils of the same quality varied with cultivation time, analyzing separately the POD activities of samples from contaminated and non-contaminated areas. It was found that, while POD activity in the roots of non-contaminated beets showed a monotonous decrease, that of the contaminated ones was lower at the beginning, increased abruptly when contamination appeared, then started to decrease again. Throughout the period investigated, however, it remained at a higher level than that of the non-contaminated samples. These observations are in good agreement with data of the literature. Although the results obtained seem convincing enough, taking into account the great number of samples and variables, the authors do not intend to draw far-reaching conclusions from these and find it necessary to carry out further investigations.

CHANGES IN MOLECULAR SIZE OF TTC-REDUCING PLASMA PROTEIN OF THE MESOPHILIC STRAIN *TORULOPSIS UTILIS* (T82) AND A PSYCHROPHILIC *CANDIDA UTILIS* YEAST STRAIN AS A FUNCTION OF CULTIVATION TEMPERATURE

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The distribution according to molecular size and TTC-reducing capacity of the fractions of plasma proteins of *Torulopsis utilis* grown at 37 and 25 °C and of *Candida utilis* grown at 25 and 5 °C in glucose medium was compared.

The cell suspensions were sonicated and plasma protein was fractionated on molecular sieve *Sephadex* G–200, in pH 7.2 Tris buffer.

Each plasma protein sample was eluted in 40 to 50 fractions. With respect to molecular size distribution, in the range of 10^5 *Daltons* one, and in the range of 10^4 *Daltons* one and two peaks, resp., were obtained.

Protein content of the fractions was determined by direct absorption UV spectrophotometry according to *Layne*. TTC reducing capacity was assessed in 0.05% TTC solution.

The results of the experiments can be summarized as follows:

TTC reducing capacity has a maximum each in the range of 10^5 as well as in that of 10^4 *Daltons*.

With both the psychrophilic and the mesophilic yeast strain molecular size pertaining to maximum reducing capacity showed a relationship, in both ranges of molecular size, with cultivation temperature.

According to the relationship, the molecular size of protein obtained at a lower growth temperature carries a lower TTC reducing capacity.

The molecular size of the protein pertaining to maximum TTC reducing capacity as determined at the optimum (37 °C for the mesophilic and 25 °C for the psychrophilic strain) and at identical cultivation temperatures (25 °C) was found to be larger for the psychrophilic than for the mesophilic strain.

This means that on decreasing cultivation temperature, the decrease occurring in the molecular size of the protein carrying maximum TTC reducing capacity reaches the minimum value yet ensuring enzyme activity earlier (*i.e.* at a higher temperature) in the mesophilic strain.

This might be an explanation of the fact why, in the case of the psychrophilic yeast strains, reducing activity can be measured also at a lower temperature (5 °C).

A CHROMATOGRAPHIC VIDEODENSITOMETRIC PROCEDURE TO STUDY TISSUE ENZYMES CATALYZING AMINO ACID TRANSFORMATION

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Since with the videodensitometer, a quantitatively evaluated thin-layer ion exchange chromatographic method, nanomole quantities of amino acids can be measured in a reproducible way (Dévényi, 1976), a new possibility has arisen to measure the activity of enzymes catalyzing amino acid transformation, in a few mg of fresh tissue sample. Reactions have been followed in which amino acids are decomposed, formed and transformed into each other. Samples taken at given intervals from the reaction mixture were chromatographed on a thin-layer ion exchange plate (Fixion 50 X 8, *Chinoin* Budapest), then the amounts of the amino acids visualized with ninhydrin were determined with the videodensitometer (Telechrom, Euro-Lab, München) (Karsai & Elődi, 1979; Karsai *et al.*, 1978).

The reaction mixture contained the substrate, co-factors, buffer and the tissue extract, in a final volume of 60 μl. The sample size of the tissue homogenate was calculated to give a difference of at least 5 nmoles in the amino

acid content of the samples taken at the beginning and at the end of the reaction. Samples of 5–10 μl taken at a given time were spotted onto marked positions of the plate, the reaction was stopped with an equal volume of 10% trichloroacetic acid. Fifty nmoles of amino acid dissolved in 5% trichloroacetic acid were spotted separately as reference. If the Rf-value of the amino acid investigated is below 0.6, trichloroacetic acid does not interfere with separation. If the Rf is near to 1, trichloroacetic acid has to be removed by preliminary chromatography in 0.01 *N* hydrochloric acid. In this step the amino acids remain at the start, while trichloroacetic acid migrates with the solvent front. Chromatography can be performed with a suitable buffer after drying of the plate. After chromatography the amino acids are detected with ninhydrin.

The chromatographic plate is placed into the videodensitometer perpendicularly to the direction of migration. The percentage amount of the amino acids formed or decomposed, is determined, whereby the total amount of the amino acids present in the samples taken at different intervals and in the reference sample are considered as 100%. From the percentage value of the reference amino acid the molar amounts of the other amino acids can be calculated. Reaction rate can be established from the time-dependence of the changes in concentration. From the rate related to the wet weight of the tissue or to the protein content of the extract, enzyme quantity (activity) can be obtained.

During the work performed, a method has been developed to determine, from different tissues, the activities of aminotransferases (aspartate-, alanine-, tyrosine-, ornithine-oxoacid-aminotransferases), amino acid dehydrogenases (glutamate and leucine dehydrogenases), urea-cycle enzymes (carbamoylphosphate synthetase, ornithine carbamoyltransferase, arginino-succinate-synthetase and lyase as well as arginase), amino acid decarboxylases (aspartate, glutamate, ornithine, histidine decarboxylases) and histidine ammonia lyase. The lowest activity detectable with the method is 20 to 30 nmoles of amino acid per h per g of wet tissue. The error of activity measurement is $\pm 5\%$.

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AUTOMATIC DETERMINATION OF AMYLASE ACTIVITY AND KINETICS

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Owing to the modernization of the starch hydrolyzing industry and to the development of new technologies, amylolytic enzymes have gained, in the last decades, an increased importance. Parallel to the increase in industrial utilization research into the configuration, action mechanism and modification of the active site affecting product distribution has been intensified with respect to above enzymes. Taking into consideration these facts, the possibility of applying continuous analyzers with amylolytic enzymes has been studied.

Making use of the elements of the Technicon Autoanalyser and the 28 channel peristaltic pump of the device Contiflo (Labor MIM), a variable manyfold has been developed which lends itself equally to quick and exact determination of amylolytic activities in great series and to the performance of kinetic measurements meeting scientific demands.

The most important task was to increase the sensitivity (0.5–3.0 g l^{-1}) of the Technicon glucose determination method (sugar determination with potassium ferricyanide) generally applied, since, in kinetic investigations, the measurement of initial rates has to be carried out at degrees of hydrolysis of only 5%. This means that the sensitivity of the method has to reach the range of 5–100 mg l^{-1} glucose.

The sensitivity of the method was increased by decreasing the potassium ferricyanide concentration and by altering the alkaline medium (0.3 *N* NaOH). The composition of the reagent was as follows:

0.15 g l^{-1} $K_3Fe(CN)_6$, 9 g l^{-1} NaCl, and 12 g l^{-1} NaOH

The method developed permitted of performing highly reproducible measurements (20 samples per h) in the glucose concentration range of 5 to 100 mg l^{-1} as proven by the corrected standard deviation $\delta = \pm 0.005$, calculated from the extinction values of 50 samples.

In activity measurements it is expedient to use a batchwise operating manyfold. Applying a constant substrate flow (1 g l^{-1} soluble starch), the enzyme solutions of different activity and protein concentration have to be placed into the sample holders.

In reaction kinetic measurements where with constant enzyme concentration and flow varying substrate concentrations (1.0–0.1 g l^{-1} starch) are required, both a batchwise operating and a continuous (gradient mixer) manyfold can be applied.

In using the latter, exponential substrate dilution was carried out. From a mixing vessel of known volume (V) concentrated initial substrate (c_o) was drawn off and a diluting buffer was added at an identical rate v_t.

Thus, actual substrate concentration c_t may be calculated at any time t from the equation below:

$$c_t = c_o \cdot e^{-v_t/V}$$

Throughout the measurements 1.0 g l^{-1} substrate concentration and 10 cm^3 mixer volume were used.

The high-sensitivity (potassium ferricyanide) method for sugar determination developed and the application of the variable manyfold permitted not only of determining in an exact way activities substantially lower than earlier, but also of performing highly reproducible kinetic measurements in the substrate concentration range around the value of K_m.

DETERMINATION OF AMYLASE ACTIVITY IN CORN WITH A CHROMOGENIC SUBSTRATE

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Corn is one of the most valuable feed-crops in Hungary. In animal breeding, the hydrolyzing effect of its tissue enzymes is of importance from the aspect of utility. That is why it was found necessary to develop a method for the determination of amylase activity in corn.

The tablet Phadebas Amylase Test, manufactured for clinical alpha-amylase activity measurements, was used as substrate.

The amylase-containing enzyme extract was prepared from corn grits. The reaction mixture of a total volume of 10 ml contained 1 ml of enzyme extract. Incubation was performed at pH 7.0, the value ensured by the tablet and 50 °C. At the end of the reaction period the enzyme was inactivated with phosphotungstic acid. Residual substrate was filtered off. Colour intensity of the reaction mixture which varied proportionally to enzyme activity was measured against a blank at 620 nm.

In developing the method attention was paid to creating reaction conditions satisfactory from the point of view of kinetics. The relationship between substrate concentration and reaction rate could be described by a sigmoidal curve. According to the curve substrate saturation could be achieved in the given reaction mixture by about 6 tablets. However, since even 3 tablets were too much to be uniformly dispersed in the reaction mixture, attempts were made at elaborating the method of measurement with 2 tablets.

In this case relationships between reaction times and optical densities proved to be linear in the concentration range of enzyme extracts between 0.1 and 0.25%. In the range investigated, reaction time varied proportionally to the concentration of the enzyme extract.

Considering these results, further experiments were carried out with 2 tablets of the Phadebas Amylase Test in a volume of 10 ml.

Substrate specificity was tested by using a beta-amylase preparation of the firm *Serva*, isolated from barley. This enzyme preparation hydrolyzed the chromogenic starch derivative contained in the tablet.

The method was applied to determine specific amylase activity in 12 corn varieties (1 Unit $= 10^{-3} \Delta$ OD $\min^{-1} g^{-1}$). Specific amylase activity was highest in the corn variety *Martonvásári* SC 480 : 260 U g^{-1}. In 7 varieties specific amylase activity was between 57 and 116 U g^{-1} and in 4 varieties between 23 and 25 U g^{-1}.

The substrate Phadebas Amylase Test lends itself to determining the specific amylase activity of corn and to establishing the differences between enzyme activities of different cultivars. In wheat somewhat higher values were obtained, up to 400 U g^{-1}, as compared to specific amylase activities found in corn.

INSTRUMENTAL MEASUREMENT OF MACERASE ACTIVITY

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An instrument has been developed at the *Bioengineering Department of the Central Food Research Institute* which permits of objective measurement of macerase enzyme action decomposing vegetable tissues.

A potato slice of 10 mm diameter and 1 mm thickness, belonging to a standard variety and applied as substrate of the enzyme macerase, was fixed between two plastic cylinders. The plastic cylinders containing the potato disk are placed in test tubes positioned in a special rack. Here they obtain the weight load. The potato slices are exposed to the pressure of a brass rod of 100 g weight and 2 mm lower diameter. After applying the weight load, the enzyme solution added to the test tube and possessing macerase activity, degrades, during the incubation period, the connective tissue between the cells which consists mainly of pectic substances. As a result of degradation the brass rod breaks through the potato slice and falls into the test tube. The cap on the upper end of the brass rod, when falling onto the rack, closes an electric circuit, *i.e.*, in closing the circuit, switches on a lamp on the back of the rack. The breakthrough of the brass rod, *i.e.* the appearance of the light of the lamp indicates the end point of the reaction.

Enzyme activity can be characterized by reciprocal reaction time and expressed as 100 min^{-1}.

The method is sensitive and reproducible if potato slices of uniform thickness are applied. On evaluating the results of measurements by mathematical statistics, the value of the variation coefficient was found to be 5.95%.

The sample size applied in instrumental analysis was 2.5 ml. Temperature optimum of macerase activity was 50 °C.

In the course of the present work two enzyme preparations have been compared, both prepared at the *Bioengineering Department of the Central Food Research Institute* by submerged fermentation of *Aspergillus awamori* and *Aspergillus foetidus*, resp. One of them is an endo-polygalacturonase of the activity of (SPA_{75}^{Na-p}): 4000 l h^{-1} g^{-1}; the other one is an apple juice clarifying enzyme complex of the apple juice clarifying activity (SPA_{75}^{A}): 137 l h^{-1} g^{-1}. pH optima of macerase activity were, for the endo-PG preparation and the apple juice clarifying PG, pH 3.5 and 3.0, resp. pH values were adjusted by means of McIlvaine buffers. When applying an incubation temperature of 40 °C and a pH value of 4.5, the curve describing macerase activity showed a second peak at pH 4.0 and 4.5, resp., corresponding to the pH optima of the hemicellulases (arabanase, xylanase) and of endo-PG.

Optimum enzyme concentration for measuring macerase activity was 0.5 mg ml^{-1}, in the case of the endo-PG preparation, and 10 mg ml^{-1}, in investigations with the apple juice clarifying preparation.

Microscopic investigations confirmed the difference in action mechanism of the two preparations. While the action of the endo-PG preparation appeared in the decomposition of potato tissue to intact individual cells, the apple juice clarifying enzyme complex degraded, beside the gum substance between the cells, also the cell walls entirely.

GRAVIMETRIC MEASUREMENT OF MACERASE ACTION

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Studies of the variation of macerase action might be important economically in processing large quantities of vegetable products of different cultivars or originating from different locations. The duration of enzymatic disintegration and the amount of the enzyme applied vary with the pH, and the pH optimum may show considerable differences according to cultivars and even locations.

A method is, therefore, needed by which optimum processing conditions can be accurately established without requiring special equipment (in control laboratories of canning factories or agricultural plants).

At the *Bioengineering Department of the Central Food Research Institute* an objective method suitable for routine analysis has been developed which determines the enzymatic disintegrating action of vegetable tissues by the decrease in weight of tissue cylinders of standard cultivar and size.

In determining macerase activity of various enzyme preparations potato tissue of uniform texture proved to be the most suitable substrate. In the present work the substrate of the enzyme was the potato cultivar *Rózsa*. Cylinders of 10 mm diameter and 20 mm length have been prepared from potatoes by means of a cork-borer and a double-bladed cutting device. Weights of the cylinders were determined with an analytical balance to four places of decimals before incubation and after 1.5 and 3 h of incubation, resp., with 5 ml of enzyme solution at 50 °C.

Macerase action is characterized by the percentage value of the weight loss of tissues as related to the original weight. Results of measurements are well reproducible (variation coefficient: 3.98%).

Optimum conditions of macerase action of the endo-polygalacturonase preparation investigated throughout the measurements (experimental product of the *Central Food Research Institute*; endo-PG activity (SPA_{75}^{Na-p}): 4000 l h^{-1} g^{-1}) were as follows: pH 3.5; temperature: 50 °C; incubation time: 3 h; enzyme concentration: 10 mg ml^{-1}.

In the course of the present work, optimum pH value of macerase activity of the endo-PH preparation was determined as a function of cultivars as well. In the case of carrots the following pH optima were obtained: *Chantenay* and *Vriflakk*: pH 3.0; *Fertődi vörös*: pH 3.5–4.0 and *Danró*: pH 3.0–4.0.

When investigating red beets, optimum pH of macerase action was found to be, in the case of the cultivars *Egyiptomi lapos* and *Bíborgömb*, pH 3.0 and 4.0, resp.

With vegetables of heterogeneous tissue texture care has to be taken to keep the ratios of the parts of different tissue texture at an identical level in the substrate cylinders.

When comparing 1.0 mg ml^{-1} concentrations of endo-PG and apple juice clarifying enzyme complex, macerase action of endo-PG proved about double that of the apple juice clarifying enzyme complex.

INVESTIGATIONS INTO THE ENZYMATIC DEGRADABILITY OF FOOD PROTEINS

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In vitro investigations applicable to the evaluation of the degradability of food and feed proteins will yield the more exact data the better they approach the enzymatic processes taking place *in vivo* in the organism. (That is

why pepsin degradation as generally used and even standardized for establishing "digestibility" of proteins cannot give utilizable results in their evaluation.) Taking into account this experience, a method of investigation has been developed which lends itself to *in vitro* routine analysis of the degradability of food and feed proteins. The steps of the method are as follows:

1. Pepsin digestion. A defatted sample of the substance to be tested, containing maximum 0.5 g of protein is hydrolyzed at 38 °C for 3 h under permanent shaking in a solution of 50 ml 0.1 *N* HCl and 1 mg pepsin (11 mAnson U mg^{-1}).

2. Trypsin digestion. After pepsin digestion the pH of the solution is adjusted to 8.4 by K_2HPO_4, then hydrolysis is continued, after addition of 1 mg of trypsin (4400 NF U mg^{-1}), for 12 h.

	N×N conversion factor	After pepsin digestion			After pepsin + trypsin digestion			After pepsin + trypsin + eripsin digestion		
		N×N conversion factor	Biuret reaction from 10 mg protein	Ninhydrin reaction from 1 mg protein	N×N conversion factor	Biuret reaction from 10 mg protein	Ninhydrin reaction from 1 mg protein	N×N conversion factor	Biuret reaction from 10 mg protein	Ninhydrin reaction from 1 mg protein
	%	%	E	E	%	E	E	%	E	E
Egg white	82.4	74.4	0.260	0.515	77.75	0.281	0.937	81.05	0.229	1.487
Whole egg protein	81.2	73.05	0.310	0.483	77.0	0.326	0.839	78.35	0.291	1.190
Powdered milk (industrial)[a]	18.75	14.25	0.293	0.375	15.75	0.349	0.629	16.25	0.314	0.884
Meat meal	53.1	30.0	0.198	0.371	30.4	0.357	0.691	37.5	0.209	0.851
Fish meal	61.5	13.5	0.143	0.284	34.5	0.317	0.412	40.5	0.306	0.916
Potatoes (freeze-dried)	11.5	8.85	0.282	0.460	9.4	0.490	0.864	10.2	0.381	1.117
Beans	21.75	15.2	0.229	0.491	18.5	0.370	0.546	17.7	0.351	1.085
Corn	7.8	4.1	0.240	0.431	4.8	0.370	0.612	5.8	0.295	0.798
Extracted soybean[b]	39.4	29.0	0.225	0.412	30.65	0.654	0.730	32.5	0.417	0.981
Extracted sunflower-seed[c]	38.8	25.8	0.257	0.426	27.4	0.403	0.606	30.3	0.725	1.133
Rapeseed	35.9	25.6	0.196	0.457	27.1	0.282	0.604	27.9	0.216	1.068
Peas	18.7	11.7	0.188	0.441	13.3	0.345	0.347	14.4	0.251	1.005
Lucerne	16.8	7.8	0.168	0.385	11.6	0.389	0.769	12.5	0.293	0.994
Yeast	42.5	22.9	0.100	0.359	24.75	0.160	0.455	29.3	0.085	0.672

[a] = N conversion factor = 6.38
[b] = N conversion factor = 5.71
[c] = N conversion factor = 5.3
E = extinction

3. Erepsin digestion. At the end of the 12th h, the pH of the solution is adjusted to 7.7 and 100 mg of erepsin (KOCH-LIGHT LAB. Ltd., Cat. No. 2451h) are added to the tryptic hydrolyzate. Erepsin digestion takes 24 h.

The progress of enzymatic degradation is checked on samples taken after each digestion period by determination of the nitrogen content in the solution, further by quantitative biuret and ninhydrin tests. Freeze-dried egg white is used to check on the procedure and whole egg protein as reference substance of enzyme action. Enzymatic degradability is best shown, after erepsin treatment, by the amount of free amino acids present in the ultrafiltrate.

Some characteristic data as obtained by the method of the author are summarized in the Table above.

It cannot be stated that by this method proteins are digested with the same efficiency as *in vivo*, however, by comparing data to those of the reference substance, *i.e.* whole egg protein, true values of measure may be obtained for a given substance by these analytical procedures.

MEASUREMENT OF THE ACTIVITY OF HYDROLYTIC ENZYMES WITH THE ACIGRAPH

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Determination of enzyme activities belongs today to routine tests of biochemical analysis. Therefore it became necessary to automate these tests or to develop methods that may be performed simply and quickly without losses of reliability.

The *Acigraph* manufactured by the firm REANAL seems to be suitable to determine the activities of hydrolytic enzymes, *e.g.* of esterases.

The basic instrument is a recording pH-meter to which a pair of generator electrodes is coupled. Thus the instrument lends itself also to coulometric titrations and can be used as a pH-stat. The firm applied the system given by *Szebellédy–Somogyi*, 1938. Electrolysis continues till the pre-set pH value is reached. As the system is able to register changes of 0.02 pH units it was assumed that it might be suitable to measure the activities of enzymes yielding an acid group or a compound containing an acid group as product of the enzymatic hydrolysis. The "endpoint" of the titration is, in this case, the pH optimum of the reaction and is pre-set on the instrument. The isotonic medium is ensured by a NaCl solution of 0.9%.

The procedure is as follows: pH optimum is set, then the substrate is dissolved in NaCl and, if necessary, pH deviations are corrected by titration. Subsequently, the substance to be tested (serum, tissue homogenate, *etc.*) is added, the instrument set to titrating position and the recorder switched on. The rate of paper displacement is 1 cm min^{-1}. When the concentration of the acid formed upon enzyme action reaches the value corresponding to 0.02 pH units, the generator electrode is switched on automatically, then, after reaching the optimum, is switched off again and this process is repeated periodically. Thus a stepwise rising curve ensues. The reaction can be continued for any time (3–5 min). Enzyme activity is equal to the cotangent of the angle enclosed by the tangent curve of the steps and the horizontal axis drawn to the origin. The value thus obtained indicates the amount, in μE, of substrate decomposed in unit time.

The method described was applied to determine acetyl cholinesterase activity in serum, liver, kidney and striated muscle as well as to assess in tissue homogenates, the activities of alkaline phosphatase, lipase, carboxyl esterase, K^+-Na^+- and Ca^{2+}-Mg^{2+}-dependent ATP-ase, resp., as well as those of arylamidase and rhodanase.

The results of cholinesterase, alkaline phosphatase as well as arylamidase measurements were checked by classical methods (*Boehringer* tests), those of rhodanase activity with an ion-selective electrode. The results were in good agreement. Adaptation of further enzyme reactions to instrumental measurement is in progress.

Since the method is simple and does not require buffer solutions, the *Acigraph* is thought to be a useful tool in laboratories, especially, where there is no possibility to purchase big and expensive automatic instruments.

APPLICATION OF ENZYMATIC GLUCOSE DETERMINATION TO THE STUDY OF ENZYME REACTIONS TRANSFORMING OR PRODUCING GLUCOSE

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Glucose determination with the glucose oxidase–peroxidase system was applied to follow the action of enzymes either catalyzing glucose producing reactions or utilizing this sugar as substrate.

An example for the first case is lactose decomposition in milk, by beta-galactosidase, to glucose and galactose. In order to follow the enzyme reaction, specific determination of one of the participating compounds is required since all three are reducing sugars. The rate of glucose formation was selected

to characterize the reaction. The initial lactose content of milk was determined by the copper reduction method prescribed by the Hungarian Standard. The (stoichiometric) amount of total monosaccharides produced from lactose and therefrom the degree of substrate conversion were calculated from the value of the quantity of glucose formed upon lactase action as determined by the glucose oxidase–peroxidase method.

The rate of depletion of the substrate glucose was used to characterize an accompanying contaminating activity of commercial glucoamylase preparations. Glucoamylase decomposes starch to glucose. The accompanying contaminating activity of the commercial preparations gives rise to the formation of reversion products from part of the glucose formed, thus impairing both yield and quality, *i.e.* purity of glucose. As the reversion products are of reducing character as well, chromatographic methods are generally used for their determination. However, the decrease in the amount of the substrate glucose may be measured considerably more rapidly and accurately with the glucose oxidase – peroxidase system which permits of expressing the reverting activity in international activity units. The dependence of the reverting activity on substrate concentration and temperature has been determined by this method.

EXPERIENCE GAINED WITH THE ENZYMATIC GLUCOSE DETERMINATION GOD(POD)PAP

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The enzymatic method is, at present, the recommended glucose determination procedure of clinical chemical laboratories. In the first step of the reaction, GOD (glucose oxidase) specifically oxidizes glucose under formation of equivalent H_2O_2 which can be determined by means of an indicator reaction. The indicator reaction may be disturbed by endogenous and exogenous substances. According to the literature (Szilágyi, 1975) the most suitable indicator reaction is given by the method of Trinder (1969) which applies phenol and 4-aminophenazone which is oxidized by POD (peroxidase), with the aid of H_2O_2, to a coloured quinoidal product. The absorption maximum of the latter is 510 nm.

This is the underlying principle of the diagnostic test GOD/POD/PAP of the firm *Galenopharm*, in use for 2 years in the laboratory of the authors. Their experience is summarized as follows. Plotting the values of optical density (y) against the concentration of known standard solutions (x = mmol dm^{-3}), the equation of the curve is: $y = 0.011 + 0.036x$; $s_{OD} = \pm 0.012$;

$r = 0.997$. Linearity can be observed up to 4000 mg dm^{-3} (22.2 mmol dm^{-3}). The standard deviation of the method is ± 17.5 mg dm^{-3} (0.097 mmol dm^{-3}). The variation coefficient is 2.16%. The method can be carried out directly and consists of the dosage of a working reagent and the sample (a detailed description is attached to the kit). The mixture can be evaluated photometrically after 20 min of incubation at 37 °C. The colour is stable for 1 h. When adapting the method, the effect of decreasing the results as caused by substances known from the literature (Teuscher & Richterich, 1971; Fox & Redstone, 1976) to disturb the indicator reaction, was investigated. Solely ascorbic acid showed linear inhibition *in vitro:* $y_{OD} = 0.191 - 0.0024\, x_{asc}$; $r = 0.987$; *i.e.* 10 mg of ascorbic acid brought about a decrease in glucose value of 12 mg.

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AUTOMATIC ENZYMATIC DETERMINATION OF STARCH

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The methods applied at present to determine the starch content of raw materials as used in agriculture and industry require extremely time-consuming preparation. Subsequent determination procedures (either polarimetric or photometric methods) do not always give unambiguous results owing to the preceding operations of digestion.

The most important part most difficult to solve in determining starch content is the dissolution (swelling) and selective hydrolysis of the starch granules of different crystal structure embedded in different ways in the cells of the various raw materials.

Several chemical and physical methods are known for the extraction (swelling) of starch granules.

Swelling of starch granules can be performed in aqueous medium between 50 and 80 °C, depending on the kind and structure of starch. The extent of swelling strongly depends on the hydrating capacity of the embedded proteins. In order to increase this, proteolytic degradation was applied, by means of a neutral protease from *Bac. subtilis*. Total dissolution of starch granules was carried out by liquefaction with alpha-amylase, then the starch thus pre-

digested and dissolved was converted to glucose with glucoamylase from *Asp. niger*. Glucose formed was measured by the ferricyanide method in the analitical module of a *Technicon* system.

Taking into account the different character of starch granules (crystal structures, embedment) three model substances were used to develop the method, *i.e.* starch, corn as well as a mixed feed. The optimum size of the granules as well as concentrations of the individual enzymes were determined in batchwise experiments.

According to the results obtained the size of the granules to be applied in continuous experiments ought to be below 250 μm, the suspension to be prepared from this grist: 0.5% and enzyme concentrations of protease and alpha-amylase 20 mg per g grist, in the case of glucoamylase 80 U per g grist. (All three enzymes are inexpensive industrial preparations. The mixture of alpha-amylase and protease is marketed under the trade name of Brew-N-zyme, its specific protease activity amounts to 300 *Northrop* U g^{-1}, its alpha-amylase activity to $1.7 \cdot 10^6$ modified *Wohlgemuth* U g^{-1}. The specific activity of the industrial glucoamylase was 100 000 U l^{-1}, the suitable enzyme was prepared from this preparation by alcoholic fractionation.)

Loosening and liquefaction were carried out in separate steps, applying the concentrations of grist and enzyme as specified above, by heating the suspension under mechanical stirring in 5 min to 80 °C and keeping it at this temperature for 7 min. After a short settling period the dissolved phase was filled into the sampler of the continuous analyzer, from where the saccharification of the sample taken by suction was carried out in a 20-min reaction spiral at 57–60 °C, by the concentration of glucoamylase already known. The amount of glucose formed was determined, in the range of 1–7 mg l^{-1} by the *Technicon* method.

This procedure permits of determining the starch contents of 20 samples of vegetable origin per h. According to the results obtained, reproducibility of the assay is satisfactory, the range of standard deviations of the starch values measured is narrow. The accuracy of the method is $\pm 1\%$.

QUANTITATIVE DETERMINATION OF PHOSPHORIC ACID ESTERS BY A RADIOMETRIC ENZYME INHIBITION METHOD

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Phosphoric acid ester type compounds are applied in large amounts in the defense against insect pests. These exert their main effect by blocking acetylcholinesterase (E.C. 3.1.1.7.). This irreversible inhibition presents a pos-

sibility to determine enzymatically the quantity of phosphoric acid esters. When incubating a given phosphoric acid ester with acetylcholinesterase, a decrease in enzyme activity takes place, depending on the character and concentration of the phosphoric acid ester as well as, exponentially, on incubation time.

Enzyme activity was determined radiometrically. Labelled ^{3}H-acetylcholine was applied as substrate. Substrate concentration was selected to be relatively high, thus the amount of the substrate transformed in unit time is proportional to enzyme activity, and further enzyme inhibition does practically not occur, as phosphoric acid esters are competitive inhibitors of acetylcholinesterase. The amount of the substrate transformed was determined, after extraction of ^{3}H-acetic acid liberated during the reaction, by liquid scintillation measurement. The reaction between acetylcholine and the enzyme was stopped by an acid buffer of high ionic strength. This promoted simultaneously the selective extraction of ^{3}H-acetic acid to the organic phase. The organic phase is the scintillation cocktail itself which is based on iso-amyl alcohol and toluene. The ^{3}H-acetylcholine preserved unchanged during the reaction remains in the aqueous phase. The low energy radiation of tritium is absorbed in the aqueous phase, thus exclusively the activity of the ^{3}H-acetic acid in the organic phase is measured. This is directly proportional to the amount of total acetic acid and thus also to enzyme activity. As after the extraction there is no need to separate the organic and the aqueous phases, all the operations, from the enzyme reactions to activity measurements, can be carried out in scintillation cuvettes. This highly increases the simplicity, rapidity and accuracy of the method. By means of the method the content of active principle of about 50 samples can be measured with slight error (0.1 ml of sample is sufficient for measurement), without enrichment, down to a level of 10^{-1}–10^{-4} ppm. The procedure lends itself to routine measurements.

The method can be well applied to decomposition measurements, since not only the initial compound, but also toxic intermediate products are measurable. This is particularly important with some phosphoric acid esters, where the enzyme inhibitory capacity of the oxidation product might be several thousand times higher than that of the initial compound. Besides, the method can be well applied to measure all the acetylcholinesterase inhibitors as well as acetylcholinesterase enzyme activity. Coupled to thin layer chromatographic separation, there is a possibility of qualitative and quantitative determination.

DETERMINATION OF THE LOWEST DETECTABLE QUANTITIES OF PHOSPHORIC ACID ESTERS AS A FUNCTION OF ENZYME ACTIVITY

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The great number and widespread application of the plant-protecting agents in use made it necessary to develop highly sensitive analytical methods for determining their residues qualitatively and quantitatively. Phosphoric acid esters and carbamates are toxic to mammals owing to their cholinesterase inhibiting property.

Cholinesterase inhibition by phosphoric acid esters and carbamates serves as a measure not only of the LD_{50} value, but also of toxicity and insecticidal activity.

Cholinesterases sensitively respond even to very small amounts of phosphoric acid esters, thus measurement of cholinesterase inhibition lends itself to determine minute quantities of these substances.

The spectrophotometric and thin-layer chromatographic enzyme inhibition method is one of the most suitable procedures to determine these insecticide residues.

The enzyme inhibition technique as combined with thin-layer chromatography has the advantage of thin-layer chromatographic separation and highly sensitive detection based on enzyme inhibition.

Interference of contaminations from plants can, in most cases, be eliminated by chromatographic separation and in such cases there is no need of preliminary separation. The method permits of determining several inhibitors simultaneously.

The qualitative identification of the plant-protecting agents can be performed by their R_f-values. Furthermore, according to the direct inhibitory or activation-dependent inhibitory character of the active principle, information can be obtained as to which group of the phosphoric acid esters it belongs to (phosphate or thiophosphate).

A drawback of the procedure is that it can be used only as a semi-quantitative method, as the use of the thin-layer spectrophotometer necessary for quantitative evaluation has not yet become general.

Owing to the great number of plant-protecting agents analyzing an unknown sample, it is not sufficient to carry out the investigations by a single method. According to the practice of the authors, the thin-layer chromatographic enzyme inhibition technique and gas-liquid chromatographic measurements mutually complete each other.

The most frequently applied enzyme sources for the thin-layer chromatographic enzyme inhibition method are different animal blood sera, various tissue homogenates (most often liver), insects, in the first place houseflies, or bee-brain homogenates and freeze-dried human or animal blood plasm. For preparing the different enzyme sources the literature describes a great number of methods, nearly every author gives another one. Thus the activity of the enzyme sources is different, according to the mode of preparation.

In developing a widely applicable routine method, the aim of the authors was to extend this to as many compounds as possible. In order to achieve this, it was indispensable to select an easily available enzyme source easy to handle and to store.

The sensitivity of the method is characterized by the LDA of the individual phosphoric acid esters (LDA = least detectable amount). For the data of LDA, differences of orders of magnitude are frequently found in the literature, even in the case of enzyme sources of the same type.

In the course of the present work comparative investigations of the enzyme sources of different origin were carried out on the basis of the activity of the enzyme.

Human plasma, horse blood serum and extracts from pig and chicken liver, resp., were used as enzyme sources.

The activities of the enzyme sources were determined by the *Elmann* method. The LDA values of five phosphoric acid esters were determined by means of the enzyme sources of known activity, but different origin, applying the same substrate, 1-naphthyl acetate. Furthermore, the influence of the substrate on the LDA value was tested. The LDA values of 15 phosphoric acid esters were determined applying the same enzyme source (freeze-dried human plasma) and 3 different substrates such as 1-naphthyl acetate, acetylcholine I and indoxyl acetate. On the basis of the results it can be stated that in the interest of reproducibility it is expedient to determine the activity of the enzyme sources for the tests.

The description of the method cannot be restricted to the method of preparation and the origin of the enzyme source, the activity of the enzyme preparation ought to be given, too, along with the determination procedure, for a comparison of the results is only possible in this way.

HIGH-SENSITIVITY DETERMINATION OF CHOLINESTERASE-INHIBITING INSECTICIDES IN FOODS BY AUTOMATIC ANALYSIS

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The spreading of the insecticides of the organophosphate type means a direct danger to humans and to warm-blooded animals as well. Automation of chemical analysis and the appearance of increasingly efficient poisons jointly created the basis for the high sensitivity automated method based on enzyme inhibition and applied by the authors to investigate the contamination of foods. The method permits of determining organophosphate contamination in 20 to 50 samples per h.

When detecting contamination, the decrease in cholinesterase activity of human lyoplasma was determined. Applying acetylthiocholine iodide as substrate and DTNB as chromogenic agent a procedure based on the principle of pre-inhibition was elaborated to be used with the *Technicon* Autoanalyzer which permitted to detect, after artificial contamination, nanogram-per-cm^3 concentrations of phosphamidone, dichlorphos and DFP in water, milk, flour, bread, fruits and commercial meat products.

The authors succeeded in detecting the contamination in all cases with good efficiency and reproducibility.

PREPARATION OF BASIC REAGENTS FOR DETECTING ANTIBODIES BY ENZYME IMMUNOLOGICAL ASSAY (E. L. I. S. A.)

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For measuring low concentrations of biologically active substances, methods based on the specificity of the antigen–antibody reaction can be applied with good results. Their advantage consists in the fact that the measurements can be carried out without purification of complex mixtures even in cases when the substance to be determined is but a very little fraction of the biological sample.

The procedure most frequently applied is the radio-immunological assay (RIA), the performance of which requires, however, a specialized laboratory, expensive instruments and the observation of strict prescriptions of labour safety and health protection. The reagent labelled with the isotope applied looses its efficiency relatively quickly.

Engwall and *Perlmann* (1971) tried to overcome the drawbacks of the RIA method as outlined above, when they introduced the enzyme-immunological assay (ELISA). The essential feature of the method is that the determination is based on enzyme activity measurement. The antigen or antibody adsorbed to a solid phase binds its reacting partner from the sample, the concentration of which can be determined on the basis of the antibody-enzyme conjugate being bound in the second step. As all compounds are able, by themselves or bound to a support, to induce antibody production in a suitable species of animals, may act as antigens, the ELISA method may be applied in a field much broader than clinical diagnostics.

Enzyme-labelled antiglobulins may be used as general reagents to detect and to determine quantitatively antibodies formed during various infections. Starting from this consideration, anti-rabbit IgG and anti-human IgGAM reagents were prepared with horse-radish peroxidase.

Covalent bond formation (*Avrameas* and *Ternynck*) as well as periodate oxidation (*Nakane* and *Kawoi*) were applied as conjugation reactions. Bifunctional compounds give rise to the formation of many by-products and enzyme utilization, in this case, is unsatisfactory. Periodate oxidation is selective, activates the carbohydrate side chains of peroxidase and the active groups react, under formation of *Schiff*-base-like bonds, with the free NH_2-groups of the antibody molecule.

According to the results of the authors, the RZ-value obtained with the bifunctional compound is lower and the yield reaches hardly one quarter of that obtained with the periodate method.

With both methods a functionally active conjugate can be obtained. On the basis of the quantitative data, the authors applied the periodate method.

The conjugates were used to detect, by the ELISA method, antibodies against tetanus. On the cavities of a polystyrol microtitrating plate 0.06 Lf tetanus toxoid was adsorbed. Onto the solid phase samples of a dilution series of anti-tetanus hyperimmune human and rabbit serum were pipetted. A similar test was carried out with corresponding negative samples. In a second step the bound antibodies linked from the added enzyme-antibody conjugate an amount proportional to their concentration. Detecting was carried out by peroxidase activity measurement. The negative samples did not bind any conjugate. The positive samples showed peroxidase activity to an extent proportional to the antibody concentration determined in experiments with animals. The amount of the bound enzyme was determined by visual reading, with the naked eye, of the colour reaction.

The ELISA method lends itself to screening tests, its performance does not require expensive equipment. If suitable antibodies are available, the determination of practically any compound is possible by this procedure.

APPLICATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY TO THE DETECTION OF PLANT VIRUSES

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The detection of virus infection in a great number of plants, especially in the fruit-, berry- and grape-bearing plants, causes a serious problem in practice, although this is one of the primary conditions of obtaining virus free propagation material. The methods of virus diagnostics employed so far in practice are either time-consuming and require field or greenhouse surfaces or are, as most serological and other biochemical methods, partly unsuitable for direct virus detection from plants of ligneous stems owing to aspecific reactions taking place on account of virus inhibitors originally present in the plants as well as to low virus concentration. A new serodiagnostical method, the enzyme linked immunosorbent assay (ELISA test) seems to be suited to avoid the above problems.

The essence of the method consists in enhancing sensitivity by linking the antibody to an enzyme. The method has been described by ENGVALL and PERLMANN (1971) for the detection of human and animal diseases, but its microplate variant for malaria has been developed by VOLLER and co-workers (1974). It has been first applied, to detect plant viruses, by VOLLER and co-workers (1976). In the short period elapsed since, the method has spread explosion-like for detecting various plant viruses, in the first place in investigation work as yet, but gradually it gains application as a routine test method in practice.

The application of the ELISA test requires, first of all, a monospecific and high titer antiserum. Immunogammaglobulin (IgG) is obtained from this by purification. Part of this is used to sensitize the polystyrene microtitrating plates, to the other part the enzyme is linked. From the titrating plates sensitized with IgG the non-adsorbed excess substance is eliminated by triplicate washing, the test sample is applied by dropping, then it is incubated and the excess is washed out again. If the sample contained an antigen, this gets bound under formation of an antibody–antigen complex. Then the enzyme-linked IgG is placed into the holes. After incubation and washing only the bound and labelled antibody remains on the surface. In case of virus positivity a "sandwich" is formed containing an antigen layer between two antibody layers. Detection of enzyme activity occurs with the enzyme substrate weighed into the holes, on the basis of the discoloration taking place. Evaluation may be performed by the naked eye or with a photometer. Colour intensity demonstrates, at the same time, virus concentration.

The enzyme applied so far in plant virology is alkaline phosphatase, the substrate is p-nitro-phenyl phosphate and the colour formed is yellow.

The investigations for the adaptation of the method have been carried out. The globular *Arabis* mosaic virus (AMV) and the rod-shaped *Sarka* virus (PPV) have been selected as test viruses (GYÖRGY, 1978a; 1978b).

AMV could be detected reliably from cucumbers as well as directly from strawberry leaves. PPV could be successfully diagnosed by the method of CLARK and co-workers (1976) from *Nicotiana clevelandii* and from peach leaves and buds, while in the case of apricot leaves, owing to their very high content of virus inhibitors, the addition of 2% polyvinyl pyrrolidone (MW 44 000) to the extracting buffer did not prove efficient. Therefore, the author recommends, for the ELISA virus test of apricot trees, to grind the leaf sample with *Polyclar* AT paste prepared with borate buffer and added in the ratio of (1 : 1). In order to increase efficiency it is expedient to perform homogenization in liquid nitrogen.

The advantage of the ELISA test over the rest of the serodiagnostical methods is that it is very sensitive, detects the virus already in concentrations of 1–2 ng ml^{-1}, is quick, does not require special equipment, is independent from the form of the virus and lends itself also to direct virus testing in plants of ligneous stems.

The introduction, in practical routine testing, of the method has been initiated by the author and it is now currently applied with fruit trees.

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Abstracts

of the papers presented at the

CONFERENCE ON FOOD MICROBIOLOGY

Budapest,

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The COMMITTEE ON FOOD SCIENCE OF THE HUNGARIAN ACADEMY OF SCIENCES, the HUNGARIAN SOCIETY OF MICROBIOLOGY and the MICROBIOLOGICAL SECTION OF THE HUNGARIAN SCIENTIFIC SOCIETY FOR FOOD INDUSTRY jointly organised a conference on microbiological aspects of food technology and of food hygiene, respectively. The conference took place at the headquarters of the HUNGARIAN ACADEMY OF SCIENCES. Abstracts of the papers read are reproduced below.

MICROBIOLOGICAL PRINCIPLES OF FOOD STABILIZATION

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Of the many alterations perishable foods may undergo (*e.g.* physical, chemical, physiological, enzymological, microbiological, hygienic, parasitological or entomological deterioration), microbiological changes are the most important ones from the point of view of national economy. The paper reviews the main groups (physical, chemical and combined methods) of procedures used for the microbiological stabilization of perishable foods. It emphasises that the choice of the method to be applied in practice must be governed by considerations not only of its antimicrobial action, but also of some possible ill-effects (nutritive value, sensory changes, *etc.*) the method may produce in the food to be stabilized. Optimisation of the method from both the microbiological and the technological points of view is a necessity.

THEORETICAL AND PRACTICAL ASPECTS OF THE BIOLOGICAL STABILIZATION OF SWEET WINES

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The biological stabilization of sweet wines, in other words the protection of bottled wine from getting cloudy, or going through an after-fermentation, may be achieved by chemical and physical techniques.

Of the preservatives, only sorbic acid and its salts are permitted to be used in wine. Factors affecting preservation, the method of application, the quantity to be used have been clarified. Although sorbic acid has been used in 36 countries, in recent years some problems necessitating further investigations have arisen. Because of the reduction of the alcohol content in wines, the permitted amount of 200 mg l^{-1} is not sufficient to ensure the protection against after-fermentation. Deficiencies in flavour may arise, too, since the above conditions promote the growth of acetic acid bacteria and some of the malolactic bacteria degrade sorbic acid to form the so called "geranium" flavour, which may not be corrected. Unfavourable changes due to bacteria may be avoided only by sufficient free sulphurous acid to inhibit their activity.

Wines are bottled leaving an air space of 5–36 ml above the liquid. The oxygen present in the air space reduces the free sulphurous acid content of the wine. To clarify the relations between reduction of the sulphurous acid content and (a) the volume of the air space, (b) the method of closing, (c) transport and (d) the potassium sorbate content, resp., extensive experiments were carried out. Results have shown that the reduction of the free sulphurous acid content depends on the volume of the air space, on the temperature of storage and on the length of transport. The method of closing and the sorbic acid content do not affect the sulphurous acid content. In the case of an air space of 10 ml, hardly any change occurred during 30 days, while in an air space of 30 ml, at 12–15 °C, the loss amounted from 10 to 15%, at 30 °C to about 40% and during an 8-h transport period to 18%. Thus, the wines should be bottled with a 10 ml air space at most and 40 mg l^{-1} free sulphurous acid content at least. To avoid flavour defects, the use of sorbic acid for preservation is not advisable, when the wine is intended to mature in bottles or kept in storage for a longer period.

The increasing capacity of wineries gives rise to new microbiological problems. Microbial cultures, causing cloudiness in wine, frequently appear in the plants. It is a basic requirement to establish microbiological laboratories capable of taxonomic investigations in addition to routine control of the individual stages of wine processing and of the keeping quality of bottled wine. The identification of the microorganisms responsible for instability, the determination of their properties (resistance to heat, alcohol, SO_2 and sorbic acid) and of the composition of the microflora facilitate the selection of the appropriate methods of protection. Investigations have shown that even heat treatment (pasteurization, hot filling) does not protect against some yeast strains. Better results may be achieved by increasing the period of heat treatment or by increasing filling temperature.

Of the physical methods membrane filtration seems to spread rapidly. This method is suitable to obtain sterile wine. Keeping the other equipments

of the bottling line sterile, biologically stable wines can be produced. In large wineries, the trend of development in this field is represented by the application of combined or individual use of heat treatment and membrane filtration as physical methods and pyro-carbonic acid dimethylester as a chemical method.

STUDY OF THE BACTERIAL SPOILAGE OF IRRADIATED POTATOES

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Data on spoilage subsequent to radiation treatment are rather contradictory. Some of the authors found little difference between spoilage in radiation treated and control tubers (Winchester, 1975; Umeda, 1977), while others observed more extensive spoilage in irradiated potatoes than in the control ones (Khan & Wahid, 1977; El-Sayed, 1977).

Potato cultivar *Somogy gyöngye* was used in the experiments in November 1977. Part of the potatoes was treated with 13.5 krad (135 Gy) and stored at 5 °C with untreated potatoes as the control. To establish spontaneous spoilage, 400 untreated and 500 irradiated tubers were studied in April 1978. More infection was found in the radiation-treated tubers than in the control samples. Considering infection by moulds only, the difference was slight (29.3% in the control and 28.8% in those treated with 135 Gy; infected tubers were expressed as percentage of the total number studied). The percentage of tubers infected by both moulds and bacteria was found higher with the radiation-treated samples than with the control (2.5% with 0 Gy and 11.8% with 135 Gy, the difference being significant at $P \geqslant 0.1\%$). Not only the number of infected tubers but also the intensity of infection was higher in the irradiated samples than in the control.

In June 1978 a batch of potatoes from the market, apparently uniform but of unknown variety, was treated with 80 Gy and subsequently inoculated with *Erwinia carotovora* var. *atroseptica* (Strain G 120) to establish their susceptibility. After a two-week incubation period the tubers were halved and the infected tissue was removed. The quantity of rotted tissue served to determine the susceptibility of the tubers. The average amount of rotted tissues in individual tubers shows the increased susceptibility of radiation-treated tubers (10.11 ± 7.05 g with 0 Gy and 18.20 ± 5.53 g with 80 Gy). The χ^2 test, at the probability level of $P = 0.1\%$, proved the difference to be significant.

The next step was to study the radiation sensitivity of *E. atroseptica* in two strains (G 120, K 459). In order to determine radiotolerance of the

strains, a suspension of about 10^7 ml^{-1} was irradiated. The surviving cell count was determined by plating. The results have shown the two strains to be of nearly equal sensitivity. The D value calculated from the survival curve was 50–70 Gy.

In further experiments, the virulence of radiation-treated *Erwinia* cells was studied. Potato variety *Desirée* was used at 0 and 100 Gy. Both samples were inoculated with the *E. atroseptica* G 120 strain, untreated and treated at 100 Gy. When untreated potatoes were inoculated with untreated bacteria average infection (in g) was substantial and the standard deviation of data was large (7.63 ± 5.69 g). In irradiated potatoes inoculated with untreated microorganisms the amount of diseased tissue was greater than with any other treatment (8.27 ± 4.20 g). The radiation-treated bacterial suspension caused greater rot in the radiation-treated tubers (7.27 ± 3.90 g) than in the control (5.16 ± 4.16 g). In this case, the difference was proven significant by the t test at the probability level of $P = 5\%$. In all the other cases, statistical analysis by the t test showed the differences to be non-significant. This was due to the large standard deviation of the data. The χ^2 distribution of the data, however, showed a higher percentage of heavily infected tubers than weakly diseased ones in the radiation-treated samples; differences were significant at $P = 0.1\%$. The lower virulence of radiation-treated bacteria may be due to the lower cell count (density of the cells was set prior to irradiation) or to the higher resistance of the control samples to the lower cell count. On the other hand, it may be due to a *de facto* lower virulence, however, this has yet to be proven. In the radiation-treated tubers this milder effect of radiation-treated bacteria was not observed, probably because of the increased sensitivity of the treated tubers. In this case the inoculum of lower cell count caused spoilage of similar extent than untreated bacteria in radiation treated potatoes.

ANALYSIS OF GROWTH KINETIC PARAMETERS IN THE PRESENCE OF ANTIFUNGAL AGENTS

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From the point of view of the food industry, it is not only the growth of microorganisms, but also the inhibition of their growth which are of great interest. Kinetic analysis of growth has been carried out mainly in order to improve growth and production indices of fermentation technologies (Holló

et al., 1978). Thus, little attention was paid to the kinetic analysis of microbial cultures proliferating under inadequate conditions. The few studies relating to this subject do not account sufficiently for the presence or the effect of antimicrobial agents in the medium (GARRET, 1971; VAN UDEN & MADEIRA-LOPES, 1970). Our kinetic studies were started on the presumption that antimicrobial agents may inhibit growth in different ways (NOVÁK & NOVÁK, 1978):

– the agent causes only cell destruction (Eqn. 1),

– the agent causes the reduction of final cell density in comparison to that of the control (${}^{i}n_{max} < {}^{0}n_{max}$); as a consequence of the reduction in the supporting capacity of the medium and/or increase the self-limiting capacity of the microbe (Eqn. 2),

– the agent reduces both the reproduction constant (${}^{i}r < {}^{0}r$) and the final cell count in comparison to that of the control; by weakening nutrient utilization, the efficiency of metabolism (Eqn. 3).

These cases related to viable cell count (n) are described by the following equations:

$$\frac{dn}{dt} = ({}^{0}r - d) \cdot \frac{{}^{d}n_{max} - n}{n_{max}} \cdot n \qquad n = \frac{{}^{d}n_{max}}{1 + \frac{{}^{d}n_{max} - {}^{0}n_{0}}{{}^{0}n_{0}} e^{-({}^{0}r-d)\cdot t}} \tag{1}$$

In turbidimetric measurements, changes of the total cell count (N = viable + dead cells) in the exponential phase, are as follows:

$${}^{d}N = {}^{0}N_{0} + \frac{{}^{0}r}{{}^{0}r - d} \cdot {}^{0}n_{0}(e^{({}^{0}r-d)\cdot t} - 1)$$

$$\frac{dn}{dt} = {}^{0}r \cdot \frac{i \cdot {}^{0}n_{max} - n}{i \cdot {}^{0}n_{max}} \cdot n \qquad n = \frac{i \cdot {}^{0}n_{max}}{1 + \frac{i \cdot {}^{0}n_{max} - {}^{0}n_{0}}{{}^{0}n_{0}} e^{-{}^{0}r\cdot t}} \tag{2}$$

$$\frac{dn}{dt} = i \cdot {}^{0}r \cdot \frac{i \cdot {}^{0}n_{max} - n}{i \cdot {}^{0}n_{max}} \cdot n \qquad n = \frac{i \cdot {}^{0}n_{max}}{1 + \frac{i \cdot {}^{0}n_{max} - {}^{0}n_{0}}{{}^{0}n_{0}} e^{-i\cdot {}^{0}r\cdot t}} \tag{3}$$

where i is the inhibition factor ($0 \leq i < 1$); d is the specific death rate; ${}^{0}n_{0}$ is the initial cell density in the control culture; ${}^{d}n_{max}$ = total final cell density in the inhibited culture

$${}^{d}n_{max} = \left(1 - \frac{d}{{}^{0}r}\right).$$

The equations were found adequate for the description of every type. In detailed studies on yeasts, the specific death rates (d), calculated from the growth curves in the presence of Miconazol, Bronopol or Sun Nit, corresponded

to the values measured in separate (fungicidal) experiments thus the validity of Eqn. 1 was proven. The validity of Eqn. 2 was proven in studies with Clotrimazol. In the presence of sorbic acid the validity of Eqn. 3 was proven if a yeast strain capable of degrading, detoxicating sorbic acid (*Candida albicans*) was applied. Including in Eqn. 3 the reduction with time of the concentration of the inhibiting agent (depending on cell count), the process of growth could be simulated with high fidelity. Of the antifungal antibiotics of the polyene type, the effect of Nystatin may be described by Eqn. 1. In contrast, the growth-inhibiting effect of Pimaricin may not be explained unambiguously by fungicid activity. This emphasizes the differences in the mechanism of action of polyenes, observed also by other authors. The effect of Pimaricin differs particularly from that of Nystatin, Candicidin and Aphotericin B, inasmuch, as even in extremely high concentrations, it does not damage the plasma membrane as manifested in ion leakage, in liberation of bound Ni^{2+}, the latter being characteristic of Pimaricin, however, reminds of Flavofungin.

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PRODUCTION OF L-LYSINE BY A MICROBIOLOGICAL METHOD

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The biological value of proteins of plant origin for feeding animals may be increased by added amino acids. Of the amino acids used for complementing feed, L-lysine is most frequently applied. A highly economical way of producing L-lysine is fermentation by threonine and methionine dependent auxotroph strains developed by mutation.

The study was aimed at a fermentation technology based on raw materials available in Hungary. Nutrients based on molasses and corn steep liquor,

most frequently used, have been replaced by carbon and bios sources. Beet molasses was successfully replaced by corn hydrolysate, hydrol, diffusion liquor from sugar manufacture, brewage and cane molasses. An attempt was made to increase lysine formation and reduce the fermentation period. A laboratory-size system was developed to perform lysine fermentation in two steps.

By stabilizing the ferment liquor through evaporation and spray drying, a lysine-containing feed concentrate was prepared. This product was improved by subsequent addition of yeast. Crystalline lysine was also prepared.

SCREENING OF WILD YEASTS TO PRODUCE MUTANTS OF HIGH METHIONINE CONTENT

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Methionine is generally considered as the second limiting essential amino acid in animal feeding. Methionine deficiency counts as a widespread chronic disease even in case sufficient amounts of protein (with an adequate proportion of protein of animal origin) are available.

In the utilization of essential amino acids, it is not the *per os* proportions that are decisive, but those present in the small intestine, at the site of absorption. Thus, in monogastric animals, only about 30–40% of the methionine is utilized.

These considerations justified the attempts at the production of yeast mutants rich in methionine. The methionine content of different yeast genera differs considerably and the scatter within one genus is also high. In *S. cerevisiae*, for instance, it is 0.5%, while in *S. carlsbergensis* it amounts to 1.0%. Thus the selection of the parent strain is of importance in producing methionine-rich varieties.

Nearly forty yeast strains were analysed recently for their methionine, raw protein and nucleic acid content.

For the preliminary selection, the methionine content was determined from the biomass by the microbiological method of *Barton-Wright*. The microorganism used was the auxotroph mutant of *Leuconostoc mesenteroides*, however, instead of the titrimetric method turbidimetry was used because of its lower standard error.

Out of the strains investigated, seven were chosen for more thorough analysis, all with methionine contents above 0.6%.

The strains were propagated in 10 repetitions with 3 parallels each and the biomass, thus obtained was analysed for methionine and raw protein

content. The mean value and the standard deviation were determined for every strain. The least significant differences were calculated on the basis of all data.

Given the uncertainty arising from the cultivation method and the average variation of each strain, a strain was considered better than the others if its mean value, obtained from 10 repetitions in 3 parallels each, surpassed the methionine content of the parent strain by at least 0.16%. The total amino acid composition of the strains was also determined in the *Aminochrom* amino acid analyzer (Labor MIM).

The essential amino acid contents related to 100 g protein were compared to the reference standard protein (FAO, 1957). *Candida guilliermondi* (No. 812) was proportionately better in 4 amino acids, while both *R. glutinis* (No. 841) and *Klyveromyces lactis* (No. 1226) showed higher values in 6 amino acids each than the reference protein.

On the basis of data obtained it appears that yeast strains No. 841 and No. 1226 are worth to be used for mutant production since their parent strains were superior.

INVESTIGATION OF LIPIDS OF INDUSTRIAL YEAST STRAINS

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In the production of bakers' yeast *Saccharomyces cerevisiae* is propagated under intense aeration. Oxygen introduction at a much higher level than earlier entailed the introduction of a new yeast strain adapted to this condition. As a consequence of intensive aeration, the type of wild yeast contaminating bakers' yeast has also changed: recently wild yeasts of highly aerobic character occur more frequently in the factory.

A comparative analysis of lipids in the biomass obtained from the industrial process used in the Budafok Bakers' Yeast Factory and the wild yeast contaminating the bakers' yeast and isolated by microbiological methods, was made. In the course of group-analysis of the lipids obtained by solvent extraction, neutral and polar lipids were separated by column chromatography. Lipid groups were studied by one and two dimensional thin-layer chromatography, then analysed for their fatty acid composition by gas chromatography.

The lipid content of the two strains (*Saccharomyces cerevisiae* VDH species and isolated wild *Candida* species) cultured under laboratory conditions was equally 3%, at the same time a substantial difference was measured in the fatty acid composition. The presence of about 30% polyene C_{18} (linoleic

and linolenic) acid in the isolated wild yeast was established and this was missing from the fatty acid composition of the industrial strain. The group analysis of lipids showed a polar lipid content of about 52–55%. In the latter fraction, the glycolipids dominate in wild yeasts, while the phospholipids prevail in the industrial strain. The major part of neutral lipids is formed by triglycerides and there is only a slight difference between the two strains. Polyene fatty acids are characteristic mainly of polar lipids in membranes, but a very high (52.5%) $C_{14:1}$ fatty acid content was observed in the sterol ester fraction of *Saccharomyces cerevisiae* VDH strain. The unusually increased palmitoleic ($C_{16:1}$) acid content (47.3% in total lipids, 36.8% in triglycerides) of the new bakers' yeast strain can be explained by the strongly aerobic conditions.

COMPARATIVE ANALYSIS OF THE MEMBRANE LIPIDS IN THE MESOPHILIC *TORULOPSIS UTILIS* VAR. VIRTANEN AND THE PSYCHROPHILIC *CANDIDA UTILIS* STRAINS

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The fatty acid composition of the membrane lipids of *Torulopsis utilis*, grown at 37 and 25 °C in glucose containing medium and of *Candida utilis*, grown at 25 and 5 °C, respectively, was compared by gas chromatography. At both temperatures, the psychrophilic strain contained a higher proportion of unsaturated fatty acids than the mesophilic strain.

At low temperatures, the function of the lipoid membrane is secured by the liquid crystalline state of the membrane lipids. This is dependent not only on the amount of unsaturated fatty acids, or on the number of unsaturated bonds in the lipids, but also on the composition of the unsaturated fatty acids.

The amount of unsaturated fatty acids was studied from several aspects. The following basic differences were found in the composition of membrane fatty acids in yeast strains of different thermoresistance:

– Lowering the growth temperature increases the amount of unsaturated fatty acids only in mesophilic microorganisms.

– The psychrophilic strain contains not only more unsaturated fatty acids but also synthesizes more fatty acids of lower carbon number (lower melting point) at both temperatures.

– In psychrophilic strains the increase in fatty acids of lower retention

volume than $C_{18:1}$ and the amount of $C_{16:1}$ occur at the expense of fatty acid $C_{18:1}$.

– The cultivation temperature causes hardly any change in the $C_{18:1}$ content in mesophilic strains.

Under the given experimental conditions, the differences in the membranes of yeast strains of varied thermoresistance may be summed up as follows: psychrophilic strains synthesize $C_{16:1}$ fatty acid without change in the amount of their unsaturated fatty acid content, while in mesophilic strains the increase in $C_{16:1}$ fatty acid is accompanied by increase in the total amount of unsaturated fatty acids.

Thus, a psychrophilic yeast strain is more effective in transforming the synthesis of its membrane fatty acids into a lipid structure of lower melting point than a mesophilic strain and this may be one explanation of their different behaviour to temperature.

ROLE OF ENTERIC BACTERIA IN FOOD HYGIENE

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Escherichia coli is still used as a faecal or sanitary indicator. The detection of other, more resistant species is complementary only. The limits as set out in standards and used in food control are the results of compromises. Development in technology should, necessarily be followed by tightening of these standards. Deterioration at a given point within the limits of the standard gives rise to the suspicion that a new source of infection entered.

Of the enteric pathogens, *Salmonellae* occur most frequently in Hungary. In the epidemiology of gastroenteritis, at least in 60% of the cases, food is the vehicle. Because of nutritional traditions, in Hungary the majority of infections is due to pork sausages and primarily to brawn. The outstanding role of pork gives rise to the necessity of raising *Salmonella*-free or specific pathogen-free (SPF) livestock in the near future. Naturally, the possibility of importing diseases with imported protein feeds, fish and bone meals and in relation to human food with imported egg-powder and other egg products, has to be accounted for.

The significance of *Shigellae* in food sanitation is but slight. However, a theoretical possibility of introducing them during processing by human impurities exists.

Of the pathogenic *Escherichia coli* groups, the importance of the serotypes causing dyspepsia in infants is also slight from the food-hygienic point of view.

Although such cases were noted in Hungary, the serotypes of invasive *E. coli* causing dysenteria are also of minor importance.

From the aspect of food hygiene it is difficult to form a clear picture of the importance of enterotoxigenic *E. coli* strains. The fact that the serological distribution of strains isolated from household animals (pig, calf or lamb) and men, deviate, further that the adhesion factors participating in genus-specific colonization in the small intestine, shows infection originating from human sources to be dangerous to human health. On the other hand, it was possible to demonstrate enterotoxin formation by a great number of pathogenic or other enteric bacteria considered apathogenic. This may be explained by plasmid-determined toxin and adhesion factor formation. In Hungary food poisoning among adults also occurred which could be traced back to the enterotoxigenic *Enterobacter cloacae* strain as the etiological agent.

In the present lack of pathology tests suitable for routine application, enteropathogenic *Escherichia coli* strains or the presence of "coliform" microorganisms in food, even within the limits of tolerance specified in standards, may endanger human health.

COMPARATIVE STUDY OF METHODS FOR THE DETERMINATION OF COLIFORM BACTERIA IN MILK

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Determination of coliform bacteria is important because their count is an index of the hygienic condition of foods. Generally the intestinal contamination is demonstrated by the detection of faecal coliform and *Escherichia coli* I bacteria by separating them from other coliforms. International standards require the determination of the total aerobic and facultative anaerobic counts as well as the coliform count (including *E. coli* I).

In these comparative studies solid and liquid nutrient media were studied in order to find the most suitable one for coliform determination. Surface cultures on *Klimmer*, modified *Drigalski* and crystal violet–neutral red–lactose–bile agar were compared. Of the liquid media, *Kessler-Swenarton*, brillant green–lactose–bile broth were compared. On the crystal violet–neutral red–lactose–bile agar medium cultures were obtained using the surface and pouring of plates technique. Results were evaluated by mathematical statistical methods. The sensitivity of media and their standard deviation were established.

CONDITIONS OF HYGIENE IN NOODLE PRODUCTION. MICROBIOLOGICAL TESTS CARRIED OUT BETWEEN 1975–1977

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The problem of bacterial contamination in noodles emerged about 10 years ago. A thorough examination throughout the country has revealed a rather unfavourable situation as regards *Staphylococcus* contamination. A *Salmonella* contamination in a large factory in Budapest has been reported elsewhere.

Sanitary regulations specify that noodles must not contain *Salmonellae*, while limits of tolerance are set for *Staphylococci*.

During the three years between 1975 and 1977 the authors investigated more than 1500 samples of noodles, about 70% of which had been imported. About 27% of the imported samples was objected to because of *Staphylococcus* contamination and about 10% was not suitable for consumption.

Hungarian products were evaluated in each plant separately. Products of small plants, industrial scale and other sources showed great differences in hygiene.

The results have shown that problems of contamination have not yet been resolved.

The main foci of contamination with *Salmonellae* and *Staphylococci* in large-scale industrial production are revealed. A final product, unobjectionable from the point of view of hygiene, may be produced only if the production technology accounts for every source of contamination.

HYGIENIC AND TECHNOLOGICAL CONSIDERATION OF YEASTS IN THE SOFT DRINK INDUSTRY

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The role of yeasts occurring in soft drinks is discussed from the ecological point of view. The ecological factors determining the fate and activity of yeasts are surveyed under the following headings:

- sources of contamination,
- intrinsic factors,

– extrinsic factors and
– implicit factors.

Out of these, the water activity, carbon, energy and nitrogen sources, pH and carbon dioxide pressure were detailed as main ecological parameters of soft drinks. Table 1 compares the average characteristics of soft drinks with the growth requirements of yeasts. It is evident that soft drinks meet the growth requirements of yeasts and provide a satisfactory ecological niche for yeast growth. Indeed, microbial spoilage of bottled soft drinks is mostly due to yeasts. Hence, the processing of soft drinks must involve some preservative treatment in order to attain good microbiological and keeping quality. Preservation can be achieved either by mild heat treatment (pasteurization) or by the addition of a preservative (*e.g.* sorbic acid). Heat destruction characteristics and growth inhibiting concentrations of some preservatives are discussed. In addition, it is a basic requirement to maintain good manufacturing practices and an efficient sanitary program throughout the soft drink producing plant. Care must be taken to prevent a build-up in population of adapted yeast strains at any point in the process. The cause of spoilage can rarely be ascribed to fruit juice concentrates or syrups used but more often it can be found in the processing line itself. Critical foci of contamination in the processing line are often the proportioning pumps, holding tanks and bottle washers, the latter particularly if returned empties are to be refilled. The rigorous hygienic inspection and routine microbiological investigation of the processing line should form part of the technology of soft drinks.

Table 1

Comparison of the average characteristics of soft drinks and the growth requirements of yeasts

Parameter	Soft drinks	Yeasts
Water (%)	80–99	a_w growth range 0.75–0.99
Sugar (%)	0.5–15.0	Fermentable carbon source
N content (%)	10^{-4}–10^{-1}	Inorganic nitrogen salts
Vitamin B (%)	traces–10^{-4}	Not required by most strains
Inorganic salts (%)	10^{-4}–10^{-1}	Potassium, phosphate
pH	2.5–4.0	Growth range 1.5–9.0 optimum 4.5–5.5
pCO_2 (kPa)	50–350	Fungistatic > 0.8 MPa

TESTING OF GENETICALLY ACTIVE SUBSTANCES IN IRRADIATED SPICES BY MICROBIOLOGICAL METHODS

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Ionising radiation at a dose level of 5 kGy is effective in reducing the viable cell count of microorganisms in spices to such an extent that they no longer contribute significantly to the microbial level of the food in which they are incorporated. After establishing technological and economic feasibility of radiation decontamination of spices, extensive studies have been initiated in Hungary to test the wholesomeness of irradiated spices under a contract with the International Project in the Field of Food Irradiation.

As a part of this program of wholesomeness testing, investigations were carried out on the possible mutagenicity of ground paprika and a spice mixture untreated or radiation treated with doses of 5 and 15 kGy, respectively. The composition of the spice mixture was as follows: paprika 55%, black pepper 14%, allspice 9%, coriander 9%, marjoram 7%, cumin 4%, and nutmeg 2%. Studies were performed using the *Ames* assay of various extracts of spices and an *in vivo* assay of urine metabolites from rats fed with a diet containing 25% spices. Urine was collected after 6 days feeding with spice-containing diets. Indicator organisms were histidine auxotroph *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100. Investigations were performed within 14 days subsequent to the radiation treatment of spices and after 90 days storage of the irradiated spices, resp. Known mutagenic substances (aflatoxin B_1, streptozotocin, α-naphthylamine and sodium azide) served as positive controls in the mutagenicity tests.

Neither samples of the spice extracts nor urine samples induced a significant increase in the frequency of revertants in the *Salmonella* test system.

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MEMBRANE LIPID COMPOSITION OF A MESOPHILIC AND A PSYCHROPHILIC YEAST

R. KEREKES and GY. NAGY

(Received March 17, 1979; accepted July 28, 1979)

The fatty acid composition of the mesophilic *Torulopsis utilis* (T_{82}) and the psychrophilic *Candida utilis* (C_9) was investigated at optimal and suboptimal cultivation temperatures. Decreasing the cultivation temperature of T_{82} from 37 °C to 25 °C resulted in the increase of the amount of total unsaturated fatty acids and that of $C_{16:1}$. On the contrary, the amount of $C_{16:1}$ increased, but the total quantity of unsaturated fatty acids did not change in the psychrophilic strain, if the cultivation temperature was shifted from 25 °C to 5 °C.

These data indicate that psychrophilic and mesophilic yeasts are adapted to lower temperatures by different mechanisms.

It is well known that psychrophilic microorganisms contain larger amounts of unsaturated fatty acids in their membrane lipids than the mesophilic ones (KATES & HAGEN, 1964; MARR & INGRAHAM, 1962; MAY CHAN *et. al.*, 1971).

It was also found that the amount of unsaturated fatty acids – first of all that of $C_{16:1}$ and $C_{18:3}$ – increased in *Candida utilis* at suboptimal cultivation temperatures (BROWN & ROSE, 1969; FARREL & ROSE, 1971; MCMURROUGH & ROSE, 1973). On the basis of these findings, however, we cannot explain the active-capable state of the membrane at lower cultivation temperatures.

In the present paper we report on our results concerning the ratios of some characteristic unsaturated fatty acids in the membranes of a mesophilic and a psychrophilic yeast, resp., at optimal and suboptimal cultivation temperatures.

1. Materials and methods

1.1. Strains

The mesophilic *Torulopsis utilis* (T_{82}) and the psychrophilic *Candida utilis* (C_9) strains were studied.

1.2. Propagation and maintenance of strains

The yeasts were cultured in a medium containing:

KH_2PO_4 0.2 g, K_2HPO_4 0.15 g, NaH_2PO_4 2.0 g, Na_2HPO_4 1.5 g, $MgSO_4 \cdot 7\,H_2O$ 0.3 g, $ZnSO_4$ 0.1 g, $(NH_4)_2SO_4$ 3.0 g, beef extract (DIFCO) 5.0 g, *Bacto* pepton (DIFCO) 4 g, yeast extract (OXOID L 21) 1 g, glucose

(anal. pure) 15 g, $FeCl_3 \cdot 6\ H_2O$ 0.1 mg, $CuSO_4 \cdot 5\ H_2O$ 0.6 mg, $MnCl_2 \cdot 4\ H_2O$ 0.15 mg, KI 0.05 mg, HBO_3 0.1 mg in 1 liter medium.

T_{82} was cultured at 37 °C and 25 °C, C_9 at 25 °C and 5 °C, resp., in 500 cm^3-Erlenmeyer flasks at pH 7. The cultures were shaken in an excentric shaker (120–130 strokes/min). The maintenance of the strains was carried out in the same medium completed with 15 g *Bacto* agar/liter (DIFCO).

1.3. Disintegration of cells

At the end of the fermentation (maximum cell count), the cells were harvested by centrifugation at $6000 \times g$ for 30 min at 5 °C. The biomass obtained was washed twice with a 0.75 % NaCl solution, then the washed pellet was suspended in the same solution (10^9/ml cell count). Disintegration was carried out in an icebath by an *MSE* MF20 type 60 W ultrasonic disintegrator at 1.5 A current for 3×10 min. After disintegration the lysate was centrifuged again at $6000 \times g$ for 30 min.

1.4. Esterification and analysis of membrane fatty acids by gas-liquid chromatography

The disintegrated cell mass was washed three times with physiological NaCl solution and centrifuged again. The pellet obtained was esterified in a methanol–benzene–sulphuric acid mixture (75 : 25 : 4). The esters were washed with saturated NaCl solution and then evaporated *in vacuo*. The dry residues of the esters were extracted by petroleum ether.

The fatty acid composition of the total lipids was determined by gas chromatography (using a model *Chromatron* GCHF 18.3-4.). Methyl esters were separated on a 198×0.635 cm in steel column packed with 10 % *Silar* SCP on 80/100 mesh *Gas Chrom* Q at 205 °C, and the eluted components were identified by comparison of retention times with those of standard compounds.

Relative proportions of the fatty acids were calculated after measuring peak height × peak width at half peak height.

2. Results

Figure 1 shows the fatty acid ester chromatograms of the mesophilic T_{82} cultured at 37 °C and 25 °C and of the psychrophilic C_9 cultured at 25 °C and at 5 °C.

The dominant role of $C_{18:1}$ can be seen except in C_9 at 5 °C, where $C_{16:1}$ proved to be the major fatty acid.

Figure 2 demonstrates that the psychrophilic strain contains more unsaturated fatty acids in the lipid fraction at both cultivation temperatures

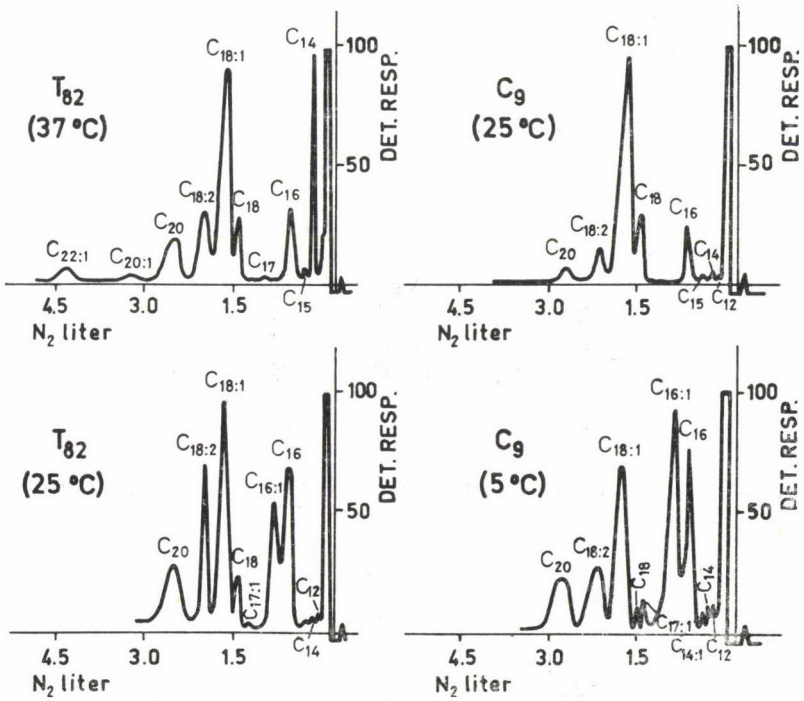

Fig. 1. Gas chromatograms of the lipids of the mesophilic strain (T_{82}) and of the psychrophilic strain (C_9) grown at the temperatures indicated

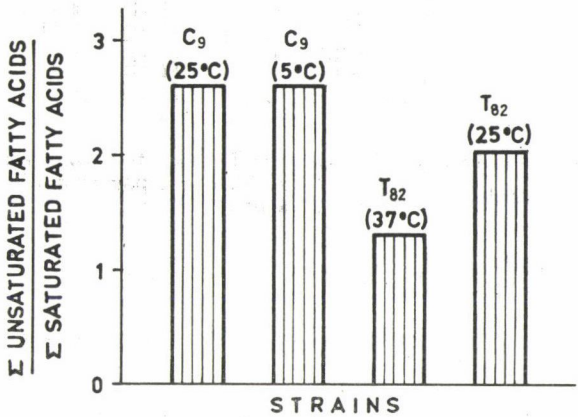

Fig. 2. The ratio of total unsaturated fatty acids: total saturated fatty acids of the mesophilic (T_{82}) and psychrophilic (C_9) strains grown at different temperatures

than the mesophilic strain. The sum of total unsaturated fatty acids versus the sum of total saturated fatty acids is equal in the psychrophilic strain at both temperatures.

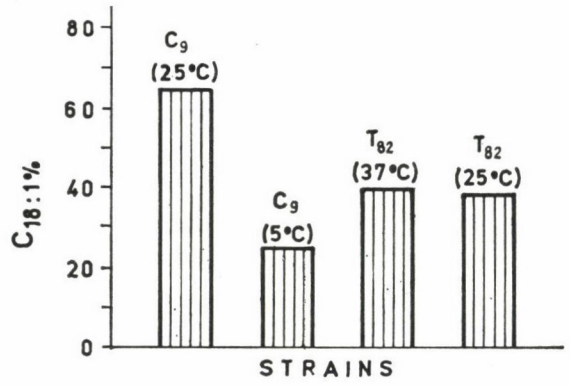

Fig. 3. Percentage of $C_{18:1}$ in the mesophilic (T_{82}) and psychrophilic (C_9) strains at different cultivation temperatures

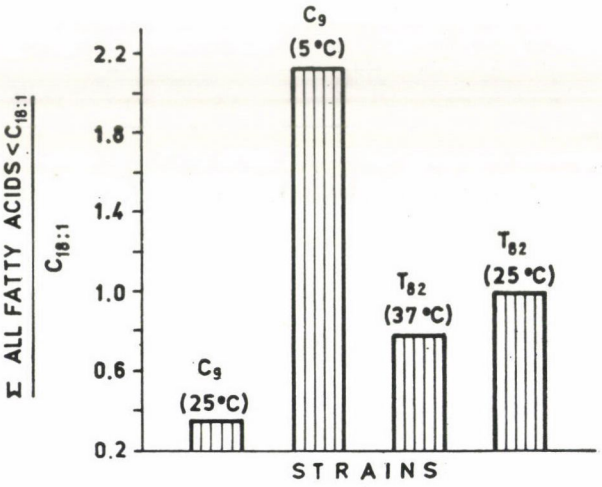

Fig. 4. Total quantities of fatty acids with smaller retention volumes than $C_{18:1}$ related to the quantity of $C_{18:1}$ in the mesophilic (T_{82}) and psychrophilic (C_9) strains grown at the temperatures indicated

This ratio of the mesophilic strain increases by about 30 % on decreasing the cultivation temperature to 25 °C.

Figure 3 shows that the percent of $C_{18:1}$ only slightly changed in the mesophilic strain, but it decreased considerably in the psychrophilic yeast, if the cultivation of the yeasts was carried out at suboptimal temperatures.

In Fig. 4 the ratios: total quantity of fatty acids with retention volumes smaller than $C_{18:1}$: quantity of $C_{18:1}$, are expressed. This ratio increased con-

siderably in the psychrophilic strain, and hardly changed in the mesophilic strain upon decreasing the cultivation temperature.

To analyse these data we determined the total amounts of monounsaturated fatty acids except $C_{18:1}$, and the quantities of $C_{16:1}$ at both cultivation temperatures of our strains. The results are shown in Fig. 5.

In the psychrophilic yeast the quantity of monounsaturated fatty acids increased from 0 to 33%, when shifting the temperature to 5 °C, and the amount of $C_{16:1}$ proved to be about 80% of the total value.

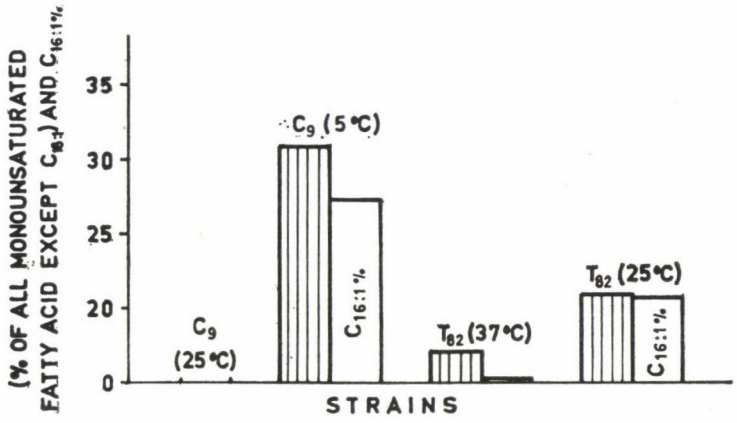

Fig. 5. Percentage of monounsaturated fatty acids except $C_{18:1}$ (striped columns) and that of $C_{16:1}$ in the mesophilic (T_{82}) and psychrophilic (C_9) strains. (The cultivation temperatures are given in parentheses)

3. Conclusions

The results of the gas-liquid chromatography experiment show that the fatty acid composition of the membrane lipids of the yeasts depend on the cultivation temperature.

The investigated psychrophilic yeast has more unsaturated fatty acids in its membrane lipids than the mesophilic one. As these results do not allow to explain the active-capable state of the membrane at lower cultivation temperatures, and as we know that the active-capable state depends not only on the amount of the unsaturated fatty acids of the membrane but also on its composition (KATES & HAGEN 1964; ROSE, 1968) we examined the changes of the dominant unsaturated fatty acids.

Our results showed that $C_{18:1}$ is the major fatty acid of T_{82} at both cultivation temperatures and of C_9 at 25 °C.

On the contrary, in C_9 strain $C_{16:1}$ proved to be the major unsaturated fatty acid, if it has been cultured at 5 °C. Many authors found that the amount

of unsaturated fatty acids, first of all that of $C_{16:1}$, increased in *Candida utilis* at suboptimal cultivation temperatures (BROWN & ROSE, 1969; FARREL & ROSE, 1971; MCMURROUGH & ROSE, 1973), but the ratios of this fatty acid to the amounts of other fatty acids have been neglected. We examined this correlation and found marked differences between the two strains.

While the amount of $C_{16:1}$ increased and the total quantity of unsaturated fatty acids didn't change in the psychrophilic strain at suboptimal temperature, the amount of $C_{16:1}$ and the quantity of total unsaturated fatty acids increased in the mesophilic strain at the lower cultivation temperature.

These data indicate that psychrophilic and mesophilic yeasts accommodate to lower temperatures by different mechanisms.

It seems that the psychrophilic strain has a more effective accommodation mechanism, as increasing the quantity of a fatty acid with a lower melting point at the expense of a fatty acid with a higher melting point might make the maintenance of the active-capable state of the membrane more possible.

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CONTRIBUTION TO THE DETERMINATION OF FUSARIUM TOXINS

Á. BATA, R. LÁSZTITY and J. BALLA

(Received September 13, 1978; accepted July 28, 1979)

The elaboration of new, more accurate methods for the determination of mycotoxin content of foods is closely connected with the improvement of extraction of mycotoxins and purification of raw extracts. Maize samples infected by *Fusarium culmorum* were extracted with ethyl acetate. After evaporation of solvents the mycotoxin-containing material was again dissolved in petroleum ether and methanol-water mixture, and purified by column chromatography. After a second purification the purified extract was investigated by thin-layer chromatography, gas chromatography and mass spectrometry. A high degree of purity was demonstrated by mass spectra.

On the basis of extended investigations, starting with the discovery of aflatoxins in 1960, more than 150 species of filamentous fungi (moulds) were reported to produce toxic secondary metabolites (mycotoxins). In recent years a number of national surveys have been carried out in various countries attempting to obtain a general pattern of the extent of food and feed contamination. Recently a good summary was given in this field by BAINTON and JONES (1977).

Under conditions in Hungary, at this time only the contamination of imported peanuts (aflatoxin) and in some cases of maize (zearalenone) has practical importance. Zearalenone [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-resorcylic acid lactone], also known as F-2 toxin, is a potent oestrogenic mycotoxin. The oestrogenic effect of zearalenone produced by different Fusarium species as reviewed by many authors (MIROCHA *et al.*, 1974; SZATHMÁRY *et al.*, 1977; *etc.*). Recent information about maize contamination by F-2 toxin was given by LÁSZTITY *et al.* (1977), BALZER *et al.* (1977), STOLOFF *et al.* (1976), COLLET & REGNIER, (1977).

The most used technique for the determination of F-2 toxin is the TLC method (EPPLEY, 1968; ROBERTS & PATTERSON, 1975; LÁSZTITY & WÖLLER, 1975; SARUDI, 1974; *etc.*).

MIROCHA and co-workers (1974) employed a solvent partition cleanup procedure and they used trimethylsilyl derivatives of zearalenone for gas-liquid chromatography with flame ionization detection. HOLDER and co-workers (1977) described a high pressure liquid chromatography (HPLC) procedure for the determination of zearalenone.

Mass spectrum of zearalenone is presented by STEELE and co-workers (1976) and SPHON and co-workers (1977).

As a part of the research programme of the Departments of Biochemistry and Food Technology, and of General and Analytical Chemistry, investigations were carried out with the purpose to improve the purification process of extracts obtained after extraction of zearalenone-containing crop samples. As a result of this investigation a modified and effective purification process was elaborated. This will be discussed in this paper.

1. Materials and methods

Maize samples infected by *Fusarium culmorum* from different parts of Hungary were used for the preparation of extracts.

1.1. Extraction of zearalenone

Ten-g samples of the infected maize were ground and extracted with 1000 cm^3 ethyl acetate in an Erlenmeyer flask for 2 hours at room temperature. The ethyl acetate extract was filtered through filter paper and was concentrated in a rotary vacuum evaporator nearly to dryness. The resulting oil was used for further purification.

1.2. Testing of purified extracts by thin-layer chromatography

Thin-layer plates of *Kieselgel* GF_{254} (0.25 mm thick) were applied. Chloroform : ethanol 95 : 5 mixture was used as a developing agent. We found that the toxin F-2 gives an intensive fluorescent spot at 0.5 R_f. Spots were visualized by spraying with a 1% solution of 4-methoxybenzene-diazonium fluoroborate in water (and 1% KOH in water). A red colour was obtained.

1.3. Testing of purified extracts by gas chromatography

The crystallized zearalenone was tested by gas chromatography. Chromatography was performed in a *Chrom* 41 type gas chromatograph with flame ionization detection. Two types of column were used: OV–17 and SE–30. The OV–17 column was 120 cm long, filled with 3% OV–17 partition liquid on a *Chromosorb* W HP (80–100 mesh). The temperature was programmed between 180–260 °C, at 4 °C min^{-1}. The SE–30 column was 300 cm long, with a stationary phase of 5% SE–30 on a *Chromosorb* W HP (80–100 mesh).

The applied temperature was 230 °C. The toxin was analyzed as its trimethylsilyl ether derivative by using *Tri-Sil* BT as silylating reagent.

1.4. Mass spectrometry of zearalenone

The mass spectra were obtained by using a JEOL IMS–OISG–2 type double focussing mass spectrometer combined with a IMA–0231/0241 mass data system. The experimental circumstances were the following:

Ion source:	electron impact
Ionizing voltage:	75 V
Ionizing current:	200 μA
Accelerating voltage:	10 kA
Electrostatic field potential:	1 kV
Pressure in the ion source:	$\sim 10^{-4}$ Pa
Pressure in the analyzer:	$\sim 10^{-5}$ Pa
Mass range:	10—500
Resolution power at low resolution operation:	1500
and at high resolution:	18 000 .

An electrical detector system was used for low resolution measurements. The high resolution measurements were taken with photo-plate detection. The solid sample to be investigated was introduced directly into the ion source.

2. Results

2.1. Elaboration of a purification process of high efficiency

On the basis of the investigation of different possibilities of purification of the extracts prepared according to para. 1.1 the following purification process was found to be the most effective.

The resulting oil was taken up with 5 cm³ benzene. This solution was purified by a *Florisil* column (4 cm diameter and 15 cm height, *Florisil* activated at 140 °C for 2 h) the zearalenone was eluted with 500 cm³ benzene, then with 1000 cm³ benzene–acetone (95 : 5). (Regeneration of the column was made by acetone.)

Figure 1 shows the elution profile of zearalenone. Fractions containing zearalenone were combined and distilled nearly to dryness. The remaining material was newly dissolved in benzene and purified by *Kieselgel* H column chromatography. (2 cm diameter, 15 cm height, *Kieselgel* H activated at 140 °C for 2 h.) Zearalenone was eluted with 200 cm³ benzene and 500 cm³ benzene–acetone (9 : 1) again. The benzene–acetone fraction was evaporated nearly to dryness in a rotary vacuum evaporator and zearalenone was

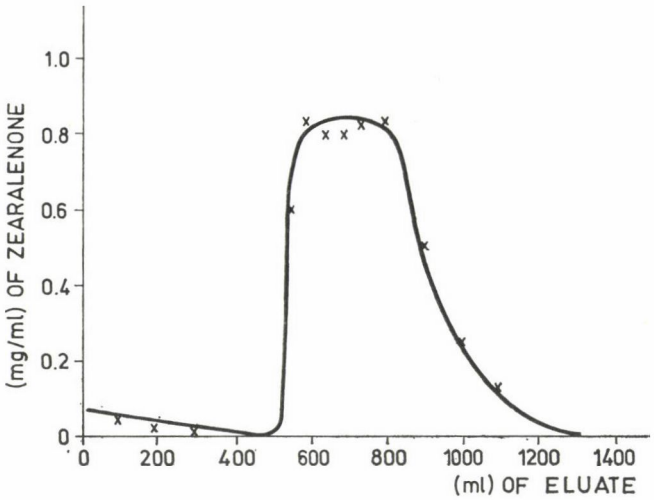

Fig. 1. Characteristic elution curve of zearalenone. (Column chromatography, *Florisil* column, elution by 500 cm^3 benzene and 1000 cm^3 benzene : acetone = 95 : 5)

crystallized from the concentrated solution. This preparation was used for further investigations.

2.2. Testing the purity of zearalenone obtained by the new purification process

The zearalenone prepared according to section 2.1 was used for TLC and GLC investigation.

In the TLC study a spot was found at $R_f = 0.5$. It was fluorescent and after developing by a spray reagent it turned red. In the GLC studies the characteristic peak of the F-2 toxin appeared at 20.3 min using OV–17 column and SE–30 column at 18.5 min.

Figure 2 shows the mass spectrum of zearalenone. In the spectrum the molecule ion 318 is well demonstrated. The exact mass of molecule ion was 318, 1465. It was measured by photoplate detection. The theoretical value based on the brutto formula $C_{18}H_{22}O_5$ is 318.

In Fig. 3 the molecular fractionation process of zearalenone is demonstrated. The characteristic peaks of the spectrum are the following:

Ion m/e = 300 formed by water loss of the molecule ion after *McLafferty*'s rearrangement; basic ion m/e = 88 and the ion m/e = 112 produced by rearrangement of ion m/e = 300.

All the results of the investigation demonstrate a high efficiency of the purification process.

Fig. 2. Mass spectrum of zearalenone

SAMPLE: REFERENCE NO.:
LEVEL: 0 DATE:
MASS RANGE: 1 810 OPERATOR:
MAX. INT.: CONDITIONS:

M/E	COEF. (SIGMA/F)	
17	107	
18	555	
27	63	
28	295	
29	69	
32	55	
39	80	
41	246	
42	51	
43	116	
44	53	
45	19	
51	36	
53	32	
54	260	
55	34	
56	67	
57	16	
59	14	
64	48	
67	96	
68	42	
69	170	
70	27	
71	84	
73	21	
74	13	
75	19	
77	74	
78	71	
79	67	
80	16	
81	93	
82	43	
83	81	
84	29	
85	15	
88	22	
91	95	
93	32	
94	17	
95	60	
96	40	
97	74	
99	16	
101	17	
102	30	
103	26	
105	31	
107	38	
109	31	
110	30	
111	20	
112	356	

Fig. 2. (Cont.)

113	121	———
115	33	——
119	46	——
121	14	—
123	25	——
124	19	—
125	262	———
126	21	—
129	71	———
131	55	———
132	30	——
133	21	—
137	21	—
147	68	———
148	42	——
149	20	—
150	32	——
151	187	———
152	19	—
159	16	—
160	69	———
161	242	———
162	74	———
163	46	——
164	61	———
165	82	———
166	12	—
175	68	———
176	147	———
177	67	———
179	65	———
185	34	——
186	30	——
187	40	———
189	343	———
191	82	———
191	17	—
192	20	—
201	20	—
202	29	——
203	102	———
204	165	———
205	54	———
206	148	———
207	138	———
228	23	—
230	13	—
231	54	———
232	20	—
249	60	———
263	39	——
281	14	—
283	27	——
300	170	———
301	27	——
318	853	———
319	173	———
320	21	—

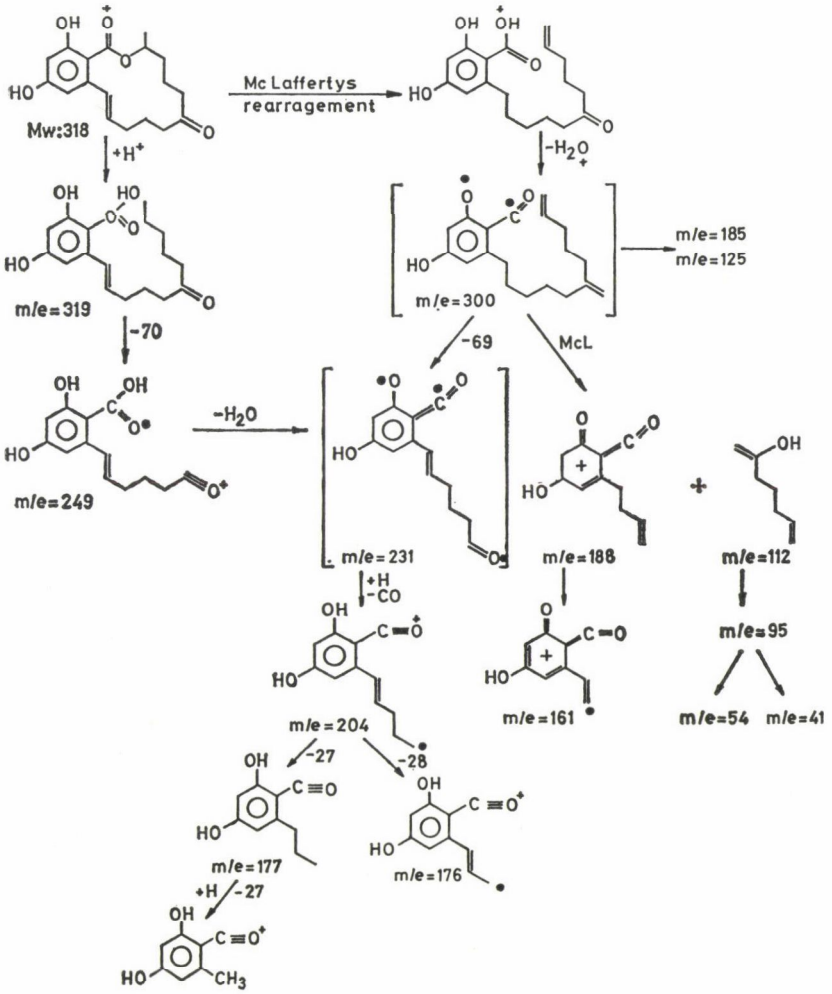

Fig. 3. Molecular fractionation process of zearalenone

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LYSINE PRODUCTION WITH *BREVIBACTERIUM* SP 22 LD USING SUGAR CANE MOLASSES

I. STUDY OF OPTIMIZATION

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A study has been made on the extracellular production of lysine with sugar cane molasses (SCM) and *Brevibacterium* sp 22 Ld (homoserine auxotroph). As a source of biosubstance, corn steep liquor (CSL) was used. By successful flask optimization, an amount of 15–20 g l^{-1} lysine could be obtained.

Further experiments were carried out in a KUTESZ fermentor (5-l medium). The results obtained showed that the optimum medium for lysine production contained: SCM (15%), CSL (3.5%), $(NH_4)_2SO_4$ (2.5%), KH_2PO_4 (0.05%) and K_2HPO_4 (0.05%) at pH = 7.0–7.5. Fermentation carried out at 30 °C for 40–50 h with 650 rpm agitation and a rate of aeration of 1.0–1.2 l l^{-1} min^{-1} afforded 35 g l^{-1} lysine.

The growing need for amino acids as pharmaceuticals and food additives has intensified the search for new sources of these materials. Previous methods for obtaining amino acids, *viz.* hydrolysis of protein (YAMADA *et al.*, 1972) and chemical synthesis (REVALLIER, 1966), both have certain disadvantages. The world shortage in proteins prevents the use of the former method, while the latter is not economical and generally yields racemates (BRECKA *et al.*, 1966).

The biosynthesis of amino acids by fermentation is of major industrial importance today. L-lysine is the only amino acid – except for L-glutamic acid – currently produced by microbial processes (HUANG, 1964). Homoserine auxotrophy in *Corynebacterium glutamicum* and *Brevibacterium* sp are the best genetic markers for lysine production (YAMADA *et al.*, 1972). *Brevibacterium* sp 22 and *Brevibacterium* sp 22 Ld were used by JANZSÓ and HOLLÓ (1977) in their studies of the kinetics of lysine fermentation. These authors used sugar beet molasses as a carbon source and corn steep liquor as a source of biological substances (thiamine, methionine, biotine, threonine). After 72 h of fermentation at pH 7–8, at 30 °C with an aeration rate of 1.5 l l^{-1} min^{-1}, 35–40 g l^{-1} lysine was obtained.

Naturally, just like in any other industrial process, economic aspects are of primary importance in choosing the raw material for a fermentation process. Sugar cane and sugar beet molasses are the most widespread and cheapest raw materials used as a major carbon source in fermentation (IMRIE, 1969). Hydrolyzates produced from cellolignins of corn husks, cotton and sunflower waste and wood were used as carbon source by DRONOV and co-workers (1967). These authors used *Micrococcus glutamicus* with $(NH_4)_2SO_4$ as a source of nitrogen at 28–30 °C and pH = 7.0 and obtained 16.1–21.8 g l^{-1} lysine. Maximum accumulation of lysine was detected after 5–6 days.

The principal aim of our investigations has been to determine an optimum medium for high lysine production from sugar cane molasses (a by-product obtained from sugar cane, the major sugar plant in Egypt).

1. Materials and methods

Sugar cane molasses (SCM) used in our experiments was obtained from the ARAB REPUBLIC OF EGYPT. Homoserine auxotroph *Brevibacterium* sp 22 Ld was used as a lysine producer. The cultures were maintained on pepton dextrose agar slants.

The inoculum was prepared by inoculating 150 cm^3 sterilized seed medium [5% SCM, 1.5% corn-steep liquor (CSL), 2.5% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4 and 1.0% $CaCO_3$, at pH = 7–7.5] in 750-cm^3 Erlenmeyer flasks with bacterial culture and incubated at 30 °C in a shaker (350 rpm) for 24 h.

Fermentation was carried out under same conditions in flasks with 10–15% inoculum for 72 h.

Further experiments were performed in a KUTESZ fermentor at 30 °C and pH = 7–7.5, with agitation at 650 rpm and an aeration rate of 1.0–1.2 l l^{-1} min^{-1}.

The fermentation media contained different concentrations of SCM and CSL, $(NH_4)_2SO_4$ 2.5%, KH_2PO_4 0.05% and K_2HPO_4. The pH value was adjusted by using $CaCO_3$ (1.0%) in flasks and NaOH (25%) in the fermentor.

Lysine content was determined by ion exchange thin-layer chromatography by the method of DÉVÉNYI (1972).

For the determination of bacterial growth, optical density (OD) was photometrically measured according to JANZSÓ and HOLLÓ (1977).

Sugar and ammonium sulfate were determined according to the methods described in the A.O.A.C. (1960).

2. Results

2.1. Flask fermentation

Table 1 shows the amounts of lysine (g l^{-1}) produced in flasks as affected by the different levels of SCM and CSL applied, 15% and 1.0%; 10% and 0.5%; 20% and 0.5%; 10% and 1.5%; 20% and 1.5%; with the former values representing SCM and the latter referring to CSL levels in the five experiments. As can be seen, there is no great difference in the amount of lysine produced at 15% or 10% of SCM. At the level of 10% SCM, the same amount of lysine (12 g l^{-1}) was produced in the presence of either 0.5% or 1.5% CSL. The amount of lysine produced at 20% SCM decreased to 8 g l^{-1} and 6 g l^{-1}

Table 1

Optimization of lysine fermentation in flasks

	N	x_0	Design		Interaction	Molasses %	Corn–steep liquor, %	Lysine g l^{-1}			
			x_1	x_2	x_1 x_2	x_1	x_2	Y'_N	Y''_N	Y'''_N	$\overline{Y}N$
Starting levels: K(x_{0i})						15	1.0	12	10	10	11
Steps						5	0.5				
Design of experiment	1	+	−	−	+	10	0.5	10	15	10	12
	2	+	+	−	−	20	0.5	7	7	10	8
	3	+	−	+	−	10	1.5	12	15	8	12
	4	+	+	+	+	20	1.5	5	8	5	6
Coefficient			−2.5*	−0.5							
New steps						2	0				
Approaching	5					15	1.0	8	9	10	9
	6					13	1.0	15	14	15	15
	7					11	1.0	14	13	15	14
	8					9	1.0	18	18	18	18
	9					7	1.0	15	14	15	15
	10					5	1.0	12	9	10	10

N = serial number of experimental variant
K = initial composition of the medium (x_{0i})
x_0 = signs needed to calculate the free number of the regression equation
x_1, x_2 (design) and x_1 x_2 (interaction) = signs required to calculate the regression coefficients
Y'_N, Y''_N, Y'''_N = results of parallel experiments
$\overline{Y}N$ = average of parallels
regression equation = $\hat{Y} = 9.5 - 2.5\ x_1 - 0.5\ x_2 - 0.5\ x_1x_2$
standard deviation of the regression coefficient = ± 0.74
* highly significant ($P > 99\%$)

in the presence of 0.5% and 1.5% CSL, resp. These results show that with low SCM concentration (10%) in the starting medium, higher amounts of lysine can be obtained at different CSL concentrations (0.5–1.5%).

A regression equation was applied as a mathematical model to estimate optimum concentrations of SCM and CSL, according to JANZSÓ (1973), and ERDÉLYI and KISS (1978). The results obtained showed a negative relationship between SCM concentration and the amount of lysine produced, whereas no such relation was observed with respect to CSL.

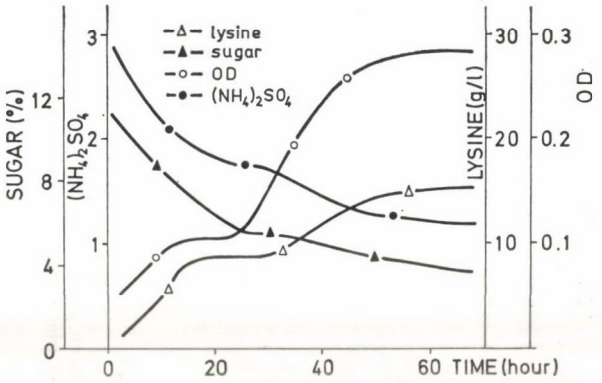

Fig. 1. Time course of lysine fermentation with 1% CSL

New flask fermentations were carried out in order to estimate the optimum concentration of SCM. In these fermentation processes only one CSL concentration, *i.e.* 1% was used. On the other hand, six concentrations of SCM, *i.e.*, 15, 13, 11, 9, 7 and 5% were applied. No other components of the media were changed. As can be seen from Table 1 the amounts of lysine produced were 9, 15, 14, 18, 15 and 10 g l^{-1} at 15, 13, 11, 9, 7 and 5% SCM concentrations, respectively. Since the highest amount of lysine (18 g l^{-1}) was obtained at 9% SCM concentration, it may be concluded that this level is the optimal concentration for lysine production.

2.2. Fermentation in a fermentor

When applied in a fermentor, the above optimum medium of flask fermentation produced only 10 g l^{-1} lysine after 71 h, as indicated in Table 2. This reduction in lysine production may be attributed to some physical and physico-chemical parameters. Therefore, SCM concentration was increased to 15%, which provided sufficient amounts of the carbon required. Concentrations of 1.0 and 2.5% CSL were examined in the presence of sufficient amounts

of carbon. Table 3 and Figs. 1 and 2 show the increase of both lysine production and bacterial growth. Similarly, the decrease in sugar and ammonium sulfate during fermentation is also given in the above Table and Figures. Although biomass production ceased at relatively low levels (0.107 and 0.150 OD at 1.0% and 2.5% CSL, resp., after 20 h), after the lapse of approximately 30 h, multiplication started again, and optical density increased to 0.286 and

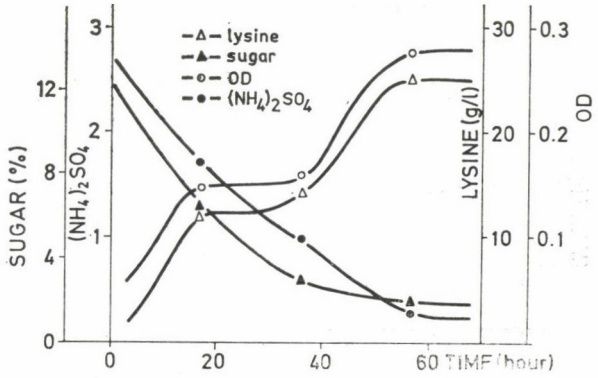

Fig. 2. Time course of lysine fermentation with 2.5% CSL

0.280 in the two fermentors after 65 h. Lysine production as well as bacterial growth took place in two stages (diauxie). The amounts of lysine obtained at 1.0% CSL concentration were 5.0, 8.8, 10.5, 13.0, 15.0 and 15.4 g l^{-1} after 10, 25, 35, 45, 55 and 65 h, resp. The corresponding lysine amounts at 2.5% CSL were 6.5, 12.0, 14.0, 18.5, 25.0 and 25.0 g l^{-1}, resp. The sugar content decreased

Table 2

Lysine produced in fermentor as a function of time, using optimum medium for flasks

No.	Fermentation time (h)	Lysine (g l^{-1})
1	2.5	—
2	6.5	—
3	22.5	2
4	28.0	—
5	32.0	2
6	46.0	7
7	50.0	8
8	54.5	9
9	71.0	10

from 11.3 to 3.7% (w/v) when the first fermentor was used. With the second fermentor, the sugar content was reduced from 12.0 to 2.0% (w/v). The corresponding reductions in ammonium sulfate in the first and second fermentors ranged from 2.9 to 1.21 and 2.8 to 0.20% (w/v), resp.

The above results point to the fact that 2.5% CSL caused higher lysine production and greater consumption of sugar and ammonium sulfate, related to 1.0% CSL. These results seem to be in good agreement with those obtained by SÁNCHEZ-MARROQUIN *et al.* (1970).

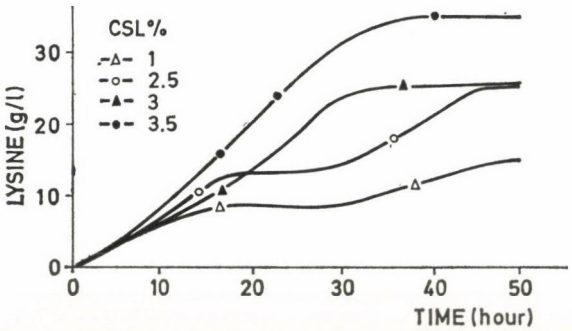

Fig. 3. Effect of different concentrations of CSL on lysine production

Table 3

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as in bacterial growth during fermentation in the presence of 1.0% and 2.5% corn-steep liquor (CSL)

No.	Fermentation time (h)	1.0% CSL				2.5% CSL			
		lysine (g l^{-1})	OD	sugar [% (w/v)]	$(NH_4)_2SO_4$ [% (w/v)]	lysine (g l^{-1})	OD	sugar [% (w/v)]	$(NH_4)_2SO_4$ [% (w/v)]
1	0	—	0.000	11.3	2.90	—	0.000	12.00	2.80
2	5	2.0	0.064	9.9	2.60	4.0	0.072	10.10	2.40
3	10	5.0	0.092	8.6	2.20	6.5	0.110	8.50	2.10
4	15	8.0	0.102	7.5	1.95	11.0	0.140	7.15	1.92
5	20	8.8	0.107	6.3	1.80	12.0	0.150	5.80	1.58
6	25	8.8	0.118	5.4	1.75	12.0	0.152	4.70	1.48
7	30	9.0	0.156	5.4	1.70	12.5	0.154	3.72	1.21
8	35	10.5	0.200	5.2	1.54	14.0	0.158	3.20	1.04
9	40	12.0	0.240	4.8	1.44	16.0	0.178	2.75	0.88
10	45	13.0	0.260	4.6	1.35	18.5	0.220	2.45	0.70
11	50	14.0	0.272	4.2	1.30	22.5	0.252	2.25	0.54
12	55	15.0	0.280	4.0	1.26	25.0	0.274	2.10	0.38
13	60	15.2	0.286	3.8	1.22	25.0	0.278	2.00	0.23
14	65	15.4	0.286	3.7	1.21	25.0	0.280	2.00	0.21

Table 4 and Figs. 3 and 4 show the effectof different concentrations of CSL on lysine production and bacterial growth. After 5 h, the amounts of lysine produced ranged between 2.5–3.0 g l^{-1}. After 20 h, lysine production greatly increased in the four fermentors as the amounts obtained were 8.0, 12.6, 12.6 and 19.5 g l^{-1}, resp. The first fermentor (1.0% CSL) afforded lysine in 8.0, 11.3 and 14.0 g l^{-1} amounts after 30, 40 and 50 h, resp. This means that no change

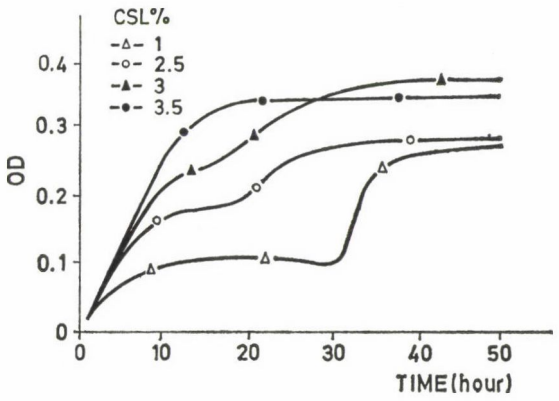

Fig. 4. Effect of different concentrations of CSL on microbial growth

could be observed in the production of lysine during fermentation for 20–30 h. With the second fermentor (2.5% CSL), however, the period of 20–25 h did not show any change in lysine production. The corresponding amounts of

Table 4

Effect of corn-steep liquor (CSL) *concentration on lysine production and bacterial growth*

No.	Fermentation time, (h)	1.0% CSL		2.5% CSL		3.0% CSL		3.5% CSL	
		lysine (g l^{-1})	OD	lysine (g l^{-1})	OD	lysine (g l^{-1})	OD	lysine (g l^{-1})	OD
1	0	—	0.000	—	0.000	—	0.000	—	0.000
2	5	2.5	0.066	2.5	0.100	2.5	0.112	3.0	0.128
3	10	5.6	0.094	6.0	0.162	5.6	0.211	7.5	0.250
4	15	7.6	0.108	10.8	0.177	9.0	0.240	13.4	0.322
5	20	8.0	0.108	12.6	0.200	12.6	0.283	19.5	0.340
6	25	8.0	0.100	12.6	0.254	17.6	0.327	25.5	0.340
7	30	8.5	0.094	13.8	0.271	23.0	0.350	31.0	0.340
8	35	10.0	0.233	16.5	0.279	25.0	0.370	33.5	0.342
9	40	11.3	0.264	20.0	0.292	25.4	0.380	35.0	0.342
10	45	13.2	0.275	24.0	0.296	26.0	0.387	35.5	0.342
11	50	14.0	0.278	24.5	0.300	26.0	0.387	35.5	0.342

lysine after 30, 40 and 50 h were 13.8, 20.0 and 24.5 g l^{-1}, resp. As regards the third (3.0% CSL) and fourth (3.5% CSL) fermentors, lysine production was found to increase as a function of time. The third fermentor yielded 23.0, 25.4 and 26.0 g l^{-1} after 30, 40 and 50 h, resp. The corresponding amounts produced by the fourth fermentor were 31.0, 35.0 and 35.5 g l^{-1}. Maximum amounts produced by the third and fourth fermentors were obtained after 45 h.

Table 4 and Figs. 3 and 4 show bacterial growth to cease at a concentration of 1.0% CSL after 10 h (0.094 OD). After the lapse of 35 h, however, vigorous growth could be observed. Optical density was the highest, *i.e.* 0.278, after 50 h. In the presence of 2.5% CSL, bacterial growth increased gradually during the first 10 h. The rate of increase was somewhat lower in the following periods, reaching 0.300 OD after 50 h.

As regards 3.0% CSL, growth was found to greatly increase against time, up to 35 h. After this period no further increase could be detected. Maximum OD, *i.e.*, 0.387, was measured after 45 h. In the fourth fermentor, at 3.5 CSL, growth was very high during the first twenty hours, and no appreciable change could be observed later on. Highest OD, 0.340 was determined after 20 h.

Characteristic diauxie appeared only at low concentrations of CSL (1.0% and 2.5%). This phenomenon can be explained by the assumption that one of the important nutrients has been consumed, therefore, in order to adapt themselves to the new conditions, the microorganisms either had to change the course of their metabolism or live on the remaining nutrients. The problem was approached by several authors and various conclusions have been drawn. According to SHIGETO (1962), after a rapid increase, growth ceased due to the preponderance of reverted cells, involving spontaneous mutation, while normal cell growth stopped due to the shortage of the growth factor. Two-stage curves point to the absence of biomaterial source. Since the nitrogen source was almost completely consumed at the end of fermentation, as presented in Table 3 $(NH_4)_2SO_4$ concentration increased from 2.5 to 3.0% in the starting medium. Fermentation was carried out in the presence of 3.5% CSL. The results obtained are summarized in Table 5 and Fig. 5.

It has been observed that under such conditions the increase in lysine production is usually accompanied by a decrease in sugar and nitrogen content. Similarly, it may be concluded that the increase in CSL concentration results in normal bacterial growth, higher lysine production, and a reduction in fermentation time. These results are in agreement with those reported by ALIKHANYAN and co-workers (1966), and JANZSÓ and HOLLÓ (1977). With optimum amount of SCM (15%) and CSL (3.5%), 35 g l^{-1} lysine was produced within 36–40 h. These results are in agreement with the findings of the KIRHENSTEIN INSTITUTE (1974) as well as the results of JANZSÓ and HOLLÓ (1977).

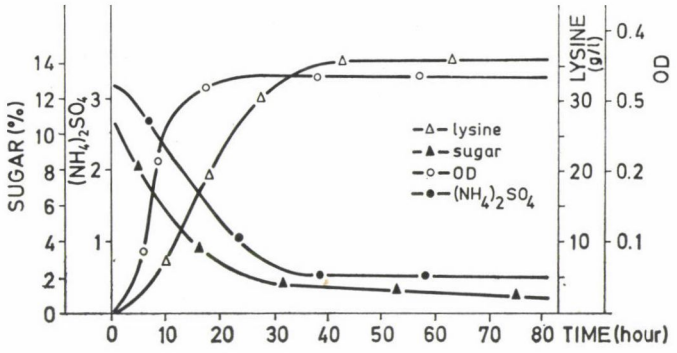

Fig. 5. Time course of lysine fermentation with 3.5% CSL

Table 5

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as bacterial growth during fermentation in the presence of 3.5% CSL

No.	Fermentation time (h)	Lysine (g l^{-1})	OD	Sugar [% (w/v)]	$(NH_4)_2SO_4$ [% (w/v)]
1	0	—	0.000	11.0	3.2
2	2.5	1	0.030	9.4	3.1
3	6.5	3	0.077	8.6	2.8
4	10.5	8	0.263	6.2	2.4
5	14.5	12	0.333	4.4	1.9
6	22.5	25	0.322	2.1	1.2
7	30.5	30	0.336	1.6	0.6
8	38.5	35	0.332	1.4	0.6
9	46.5	35	0.295	1.3	0.6
10	54.5	35	0.310	1.2	0.6
11	72.0	35	0.325	1.0	0.6

3. Conclusions

SCM proved to be an efficient carbon source in the case of *Brevibacterium* sp 22 Ld for the microbial production of lysine.

By this microbial method considerable amount of lysine can be produced, which may be of great benefit to some sugar cane producing countries as Egypt, Cuba and Nigeria, *etc.*

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LYSINE PRODUCTION WITH *BREVIBACTERIUM* SP 22 LD USING SUGAR CANE MOLASSES

II. EFFECT OF CARBON SOURCE CONCENTRATION, pH AND RATE OF AERATION

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Problems concerning the effect of carbon source concentration, pH and rate of aeration on the production of lysine by *Brevibacterium* sp 22 Ld were observed in a 10-l fermentor containing 5 l of the medium. Best lysine production was achieved on a medium containing 15% sugar cane molasses (SCM) at pH of 6.8–7.3 and an aeration rate of 0.6–1.0 l l^{-1} min^{-1}.

As it is known, most proteins of plant origin are poorer in lysine than animal proteins. The biological value of plant proteins can be raised by the addition of amino acids. L-lysine is used today all over the world to complete the feed of monogastrics. A preliminary report on L-lysine production, using sugar cane molasses and homoserine auxotroph of *Brevibacterium* sp 22 Ld was published in a previous paper (KHALAF ALLAH *et al.*, 1980). The yield of lysine varied significantly, depending on the molasses and corn-steep liquor used for the preparation of the nutrient medium (ALIKHANYAN *et al.*, 1966). The kinetics of biomass formation and lysine accumulation by the mutant of *Brevibacterium* sp 22 depended on the quality and quantity of the seeding material. The economical coefficient of lysine synthesis depended on the initial concentration of sugar in the medium (MEZINA & CEDERE, 1972). Oxygen supply to aerobic cultures was very important for optimization of the fermentation process. SELGA and BEKER (1972) studied the effect of aeration on the biosynthesis of L-lysine by *Brevibacterium* sp 22 under batch fermentation conditions. It was shown that by changing the aeration conditions it was possible to direct the process towards either biomass accumulation or a more intensive synthesis of lysine (MEZINA, 1968). In a pilot plant scale operation a new improved method of lysine production was tested (BEKERS *et al.*, 1969). In this study $CaCO_3$ was replaced by NH_4OH to adjust the pH values of the medium (molasses, corn-steep liquor, KH_2PO_4 and K_2HPO_4). Fermentation was carried out at 22–30 °C, pH = 6.9–7, and 45–60 mg O_2 l^{-1} min^{-1} aeration rate. The effect of different aeration intensities of 40–75 mg O_2 l^{-1} min^{-1} on the growth of lysine producers *Brevibacterium* sp 22 was found to depend on the concentration of molasses in the culture medium (MEZINA, 1975).

The aim of the present work was, therefore, to study the effect of sugar cane molasses concentration, pH value and rate of aeration in lysine fermentation.

1. Materials and methods

The microorganisms applied and analytical methods of lysine, biomass, sugar and ammonium sulfate were described in the previous paper (KHALAF ALLAH *et al.*, 1980). The different experimental methods used will be discussed in subsequent sections.

2. Results

2.1. Effect of carbon source concentration

Table 1 and Fig. 1 show the effect of the concentration of sugar cane molasses (SCM) (15 and 20%) on lysine production. The increase in both lysine biosynthesis and bacterial growth, as well as the decrease in sugar and ammonium sulfate during fermentation are presented in the above Table and Figures. At 15% SCM concentration the amounts of lysine obtained were 18.0, 26.0, 28.0, 33.0, 35.0 and 35.0 g l^{-1} after 20, 25, 30, 35, 40 and 45 h, respectively. The corresponding amounts of lysine at 20% SCM concentration were 12.0, 18.0, 22.0, 25.0, 25.0 and 28.0 g l^{-1}, resp. Intensive and regular bacterial growth was observed at 20% SCM concentration. Optical density was highest (0.565) after 35 h. Consequently, sugar and nitrogen (ammonium sulfate) decreased, 83.53% and 86.02% sugar and 86.31% and 89.73% ammonium sulfate were consumed at 15 and 20% SCM concentrations, resp., after 45 h. It may be

Table 1

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as bacterial growth

Fermentation time, h	Lysine, g l^{-1}	OD	20% SCM					
			Invert sugar			$(NH_4)_2SO_4$		
			g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0	0.000	138.80	100	0.00	26.30	100	0.00
0	0	0.015	—	—	—	—	—	—
5	1	0.100	119.80	86.31	13.69	23.80	90.49	9.51
10	7	0.135	103.70	74.71	25.29	21.50	81.75	18.25
15	10	0.152	90.40	65.13	34.87	17.90	68.06	31.94
20	12	0.245	84.80	61.10	38.90	13.60	51.71	48.29
25	18	0.420	60.50	43.59	56.41	8.20	31.18	68.82
30	22	0.510	32.80	23.63	76.37	2.90	11.03	88.97
35	25	0.565	21.38	15.40	84.60	2.70	10.27	89.73
40	25	0.520	19.40	13.98	86.02	2.70	10.27	89.73
45	28	0.485	19.4	13.98	86.02	2.70	10.27	89.73

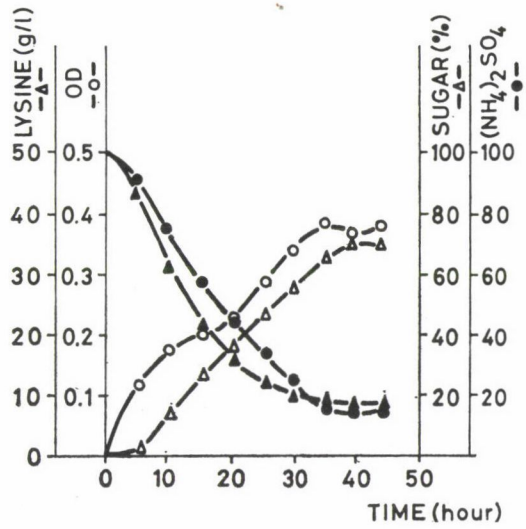

Fig. 1. Time course of lysine fermentation with 15% SCM

observed that the increase in lysine production usually involves a decreast in both sugar and nitrogen content. Similarly, it may be concluded thae 15% SCM concentration results in higher lysine production and lower bacterial growth, whereas 20% SCM concentration causes lower lysine production and

during fermentation in the presence of 15% and 20% SCM

Lysine, g l^{-1}	OD	15% SCM					
		Invert sugar			$(NH_4)_2SO_4$		
		g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0.000	105.80	100	0.00	26.30	100	0.00
0	0.015	—	—	—	—	—	—
1	0.118	90.40	85.44	14.56	23.60	89.73	10.27
8	0.185	67.48	63.78	36.22	19.70	74.90	25.10
13	0.200	50.36	47.60	52.40	15.40	58.56	41.44
18	0.235	32.80	31.00	69.00	12.20	46.39	53.61
26	0.275	25.34	23.95	76.05	9.80	37.26	62.74
28	0.340	22.70	21.46	78.54	6.40	24.33	75.67
33	0.385	18.74	17.71	82.29	3.60	13.69	86.31
35	0.367	17.42	16.47	83.53	3.60	13.69	86.31
35	0.385	17.42	16.47	83.53	3.60	13.69	86.31

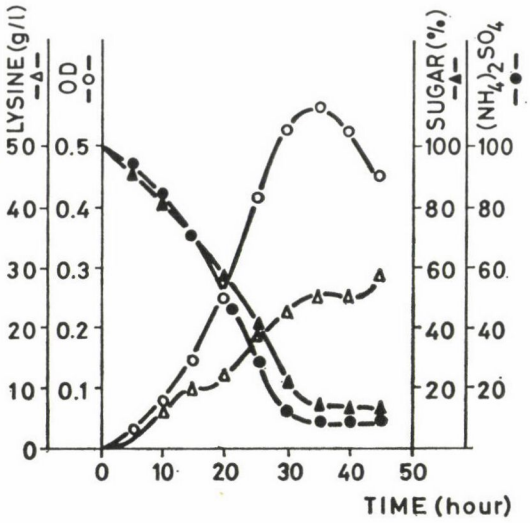

Fig. 2. Time course of lysine fermentation with 20% SCM

higher bacterial growth. In agreement with our results ARESHKINA *et al.* (1965) also found 15% SCM to be the best concentration leading to highest L-lysine production (25 g l^{-1}).

Table 2a

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as bacterial

Fermentation time, h	Lysine, g l^{-1}	OD	pH 8.3–7.8					
			Invert sugar			$(NH_4)_2SO_4$		
			g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0	0.000	110.08	100	0.00	25.89	100	0.00
0	0	0.005	—	—	—	—	—	—
5	1	0.125	99.50	90.39	9.61	23.91	92.35	7.65
10	4	0.155	80.60	73.22	26.78	19.95	77.06	22.94
15	8	0.205	55.72	50.62	49.38	16.78	64.91	35.19
20	12	0.235	47.06	42.75	57.25	13.48	52.07	47.93
25	20	0.250	28.04	25.47	74.53	11.88	45.89	54.11
30	20	0.232	18.08	16.42	83.58	9.90	38.24	61.76
35	22	0.315	18.08	16.42	83.58	7.39	28.54	71.46
40	27	0.372	15.44	14.03	85.97	4.22	16.30	83.70
45	30	0.372	11.52	10.47	89.53	4.22	16.30	83.78

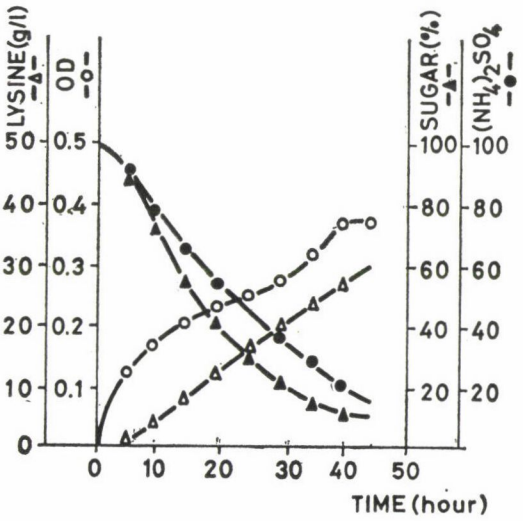

Fig. 3a. Time course of lysine fermentation at pH 7.8–8.3

2.2. *Effect of pH value*

For the determination of optimum pH value suitable for bacterial growth and for the production of sufficient amounts of lysine, fermentation was carried out at pH 8.3–7.8, 7.8–7.3, 7.3–6.8 and 6.8–6.3 while the other factors were maintained at the same level.

growth during fermentation at different pH values

Lysine, g l^{-1}	OD	pH 7.8–7.3					
		Invert sugar			$(NH_4)_2SO_4$		
		g l^{-1}	Residual	Consumed	g l^{-1}	Residual	Consumed
			%			%	
0	0.000	110.08	100	0.00	25.89	100	0.00
0	0.006	—	—	—	—	—	—
1	0.130	99.50	90.39	9.61	22.06	85.21	14.79
4	0.170	88.30	80.21	19.79	19.55	75.51	24.49
9	0.203	57.08	51.85	48.15	15.20	58.71	41.29
14	0.227	49.04	44.55	55.45	12.41	47.93	52.07
22	0.252	28.04	25.47	74.53	10.30	39.78	60.22
25	0.260	27.36	24.85	75.15	8.05	31.09	68.91
27	0.365	23.36	21.22	78.78	4.75	18.35	81.65
33	0.385	17.42	15.82	84.18	3.17	12.24	87.76
35	0.385	13.52	12.28	87.72	3.17	12.24	87.76

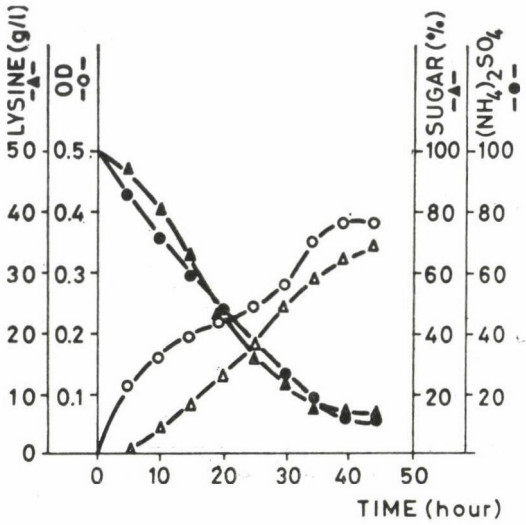

Fig. 3b. Time course of lysine fermentation at pH 7.3–7.8

The results are presented in Tables 2a and 2b and in Figs. 3a, 3b, 3c, 3d. As can be seen from the data, optimum lysine production (38–40 g l^{-1}) was obtained at pH = 6.8–7.3. Bacterial growth was also found to be highest at these pH values. No marked difference could be observed, on the other hand,

Table 2b

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as

Fermentation time, h	Lysine, g l^{-1}	OD	pH 7.3–6.8					
			Invert sugar			$(NH_4)_2SO_4$		
			g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0	0.000	111.56	100	0.00	25.96	100	0.00
0	0	0.032	—	—	—	—	—	—
5	2	0.193	80.60	72.25	27.75	20.67	79.62	20.38
10	15	0.247	53.68	48.12	51.88	17.74	68.34	33.66
15	20	0.310	24.36	21.84	78.16	12.18	46.92	53.08
20	30	0.378	19.40	17.39	82.61	8.62	33.20	66.80
25	30	0.410	19.40	17.39	82.61	6.11	23.53	76.47
30	30	0.497	14.12	12.66	87.34	6.11	23.53	76.47
35	35	0.495	11.52	10.33	89.67	6.11	23.53	76.47
40	37	0.462	10.88	9.75	90.85	6.11	23.53	76.47
45	40	0.490	10.24	9.34	90.66	5.96	22.96	77.04

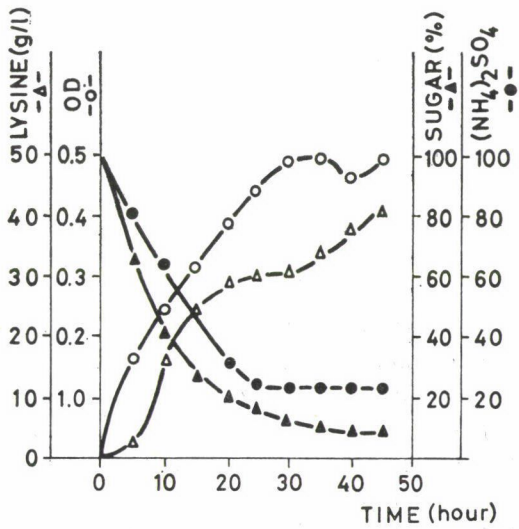

Fig. 3c. Time course of lysine fermentation at pH 6.8–7.3

in the sugar and ammonium sulfate consumption at the different pH values studied.

For pH adjustment, NaOH (25%) and NH_4OH were used throughout the experiments. In case (NH_4OH) served as the nitrogen source, 1% of the main nitrogen source $(NH_4)_2SO_4$ was applied instead of 2.5% in the basic fermentation media.

bacterial growth during fermentation at different pH values

Lysine, g l^{-1}	OD	pH 6.8–6.3					
		Invert sugar			$(NH_4)_2SO_4$		
		g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0.000	113.92	100	0.00	25.96	100	0.00
0	0.022	—	—	—	—	—	—
1	0.198	81.30	71.37	28.63	20.99	80.86	19.14
13	0.246	59.80	52.49	47.51	16.82	64.79	32.21
18	0.265	45.04	39.53	60.47	14.18	54.62	45.38
25	0.312	24.02	21.08	78.92	10.99	42.33	57.67
29	0.367	21.38	18.77	81.23	7.69	29.62	70.38
29	0.455	16.76	14.71	85.29	6.11	23.53	76.47
32	0.500	14.12	12.39	87.61	6.11	23.53	76.47
36	0.482	11.52	10.11	89.89	6.11	23.53	76.47
38	0.485	10.24	8.99	91.01	6.11	23.53	76.47

Table 3a

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as bacterial

Fermentation time, h	Lysine, g l^{-1}	OD	0.6 l air l^{-1} min^{-1}					
			Invert sugar			$(NH_4)_2SO_4$		
			g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0	0.000	99.00	100	0.00	27.95	100	0.00
0	0	0.023	—	—	—	—	—	—
5	0	0.160	95.60	96.57	3.43	24.82	89.12	10.88
10	4	0.290	70.10	70.80	29.20	10.99	39.46	60.54
15	10	0.340	51.80	52.32	47.68	10.99	39.46	60.54
20	17	0.397	40.25	40.66	59.34	7.39	26.54	73.46
25	30	0.340	25.60	25.86	74.14	4.79	17.20	82.80
30	32	0.374	17.60	17.78	82.22	2.58	9.26	90.74
35	32	0.315	17.60	17.78	82.22	3.04	10.92	89.08
40	34	0.327	16.00	16.16	83.34	2.17	7.79	92.21
45	34	0.395	16.00	16.16	83.84	2.17	2.17	92.21

Table 3b

Changes in lysine, sugar and $(NH_4)_2SO_4$ content as well as bacterial

Fermentation time, h	Lysine, g l^{-1}	OD	1.4 l air l^{-1} min^{-1}					
			Invert sugar			$(NH_4)_2SO_4$		
			g l^{-1}	Residual %	Consumed %	g l^{-1}	Residual %	Consumed %
0	0	0.000	109.20	100	0.00	29.38	100	0.00
0	0	0.024	—	—	—	—	—	—
5	0	0.152	60.05	54.99	45.01	26.53	90.30	9.70
10	2	0.192	33.65	30.82	69.18	24.16	82.23	17.77
15	6	0.206	33.65	30.82	69.18	15.46	52.62	47.38
20	10	0.225	33.65	30.82	69.18	11.97	40.47	59.53
25	14	0.203	27.20	24.91	75.09	11.52	39.21	60.79
30	20	0.283	25.60	23.44	76.56	8.28	28.18	71.82
35	22	0.261	24.00	21.98	78.02	6.11	20.80	79.20
40	24	0.302	20.80	19.05	80.95	5.00	17.02	82.98
45	28	0.352	14.40	13.19	86.81	4.56	15.52	84.48

growth during fermentation in the presence of 15% and 20% SCM

Lysine, g l^{-1}	OD	1.0 l air l^{-1} min^{-1}					
		Invert sugar			$(NH_4)_2SO_4$		
		g l^{-1}	Residual	Consumed	g l^{-1}	Residual	Consumed
			%			%	
0	0.00	99.00	100	0.00	27.85	100	0.00
0	0.023	—	—	—	—	—	—
0	0.185	90.50	91.41	8.59	22.19	79.68	20.32
7	0.300	63.55	63.99	36.01	16.09	57.77	42.23
17	0.348	40.25	40.66	59.34	10.99	39.46	60.54
18	0.357	38.60	38.99	61.01	5.22	18.74	81.26
32	0.342	35.30	35.66	64.34	4.79	17.20	84.34
32	0.319	27.20	27.47	72.53	4.79	17.20	82.80
32	0.295	27.20	27.47	72.53	4.36	15.66	84.34
35	0.319	24.00	24.24	75.76	4.36	15.66	84.34
35	0.385	14.40	14.55	85.45	4.36	15.66	84.34

growth during fermentation in the presence of 15% and 20% SCM

Lysine, g l^{-1}	OD	1.8 l air l^{-1} min^{-1}					
		Invert sugar			$(NH_4)_2SO_4$		
		g l^{-1}	Residual	Consumed	g l^{-1}	Residual	Consumed
			%			%	
0	0.000	105.80	100	0.00	30.89	100	0.00
0	0.032	—	—	—	—	—	—
0	0.075	82.00	77.50	22.50	27.87	90.22	9.78
1	0.255	61.70	58.32	41.68	24.59	79.61	20.39
6	0.325	61.70	58.32	41.68	18.57	60.12	39.88
8	0.312	43.53	41.14	58.86	10.54	34.12	65.88
14	0.353	38.50	36.39	63.61	8.54	27.65	72.35
18	0.350	35.30	33.36	66.64	4.79	15.51	84.49
18	0.397	28.80	27.22	72.78	4.53	14.66	85.34
24	0.480	28.80	27.22	72.78	4.28	13.88	86.14
24	0.460	27.20	25.11	74.29	4.28	13.88	86.14

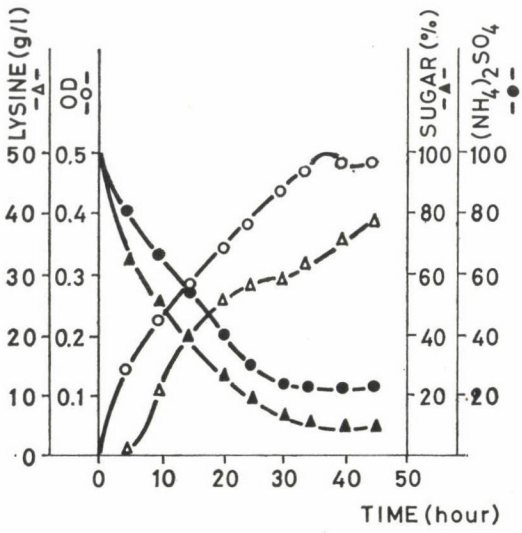

Fig. 3d. Time course of lysine fermentation at pH 6.3–6.8

2.3. Aeration effect

Fermentation (5 l medium) was carried out at different aeration rates, *i.e.* 0.6, 0.1, 1.4 and 1.8 l air l^{-1} min^{-1}. Tables 3a and 3b and Fig. 4 show the dependence of lysine concentration and biomass under various conditions of aeration. The amount of lysine produced at the above aeration levels after 45 h were 34, 35, 28 and 24 g l^{-1}, respectively. The efficiency of lysine production (g lysine/100 g sugar consumed) proved to be 40.96, 41.37, 29.54 and 30.53 %. The corresponding optical densities indicating bacterial growth were 0.395, 0.385, 0.352 and 0.460, respectively. Lysine production and bacterial growth are usually accompanied by a decrease in sugar and nitrogen content under the conditions studied. It has been observed that low aeration rates lead to an increase in lysine production, whereas higher aeration rates result in an increase in bacterial growth. These results are in agreement with those reported by BEKER *et al.* (1973). Similarly, it may be concluded that 0.8 l air l^{-1} min^{-1} was the optimum aeration level for lysine production. This aeration rate at 650 rpm ensures about 38 mg O_2 l^{-1} min^{-1}. According to ARESHKINA *et al.* (1965). the most suitable aeration conditions for the production of highest L-lysine yields (25 g l^{-1}) are: 1 vol. of air/vol. of liquid/min.

3. Conclusions

It has been established that there is a correlation between lysine production by *Brevibacterium* sp 22 Ld and carbon source concentration, the pH value and rate of aeration.

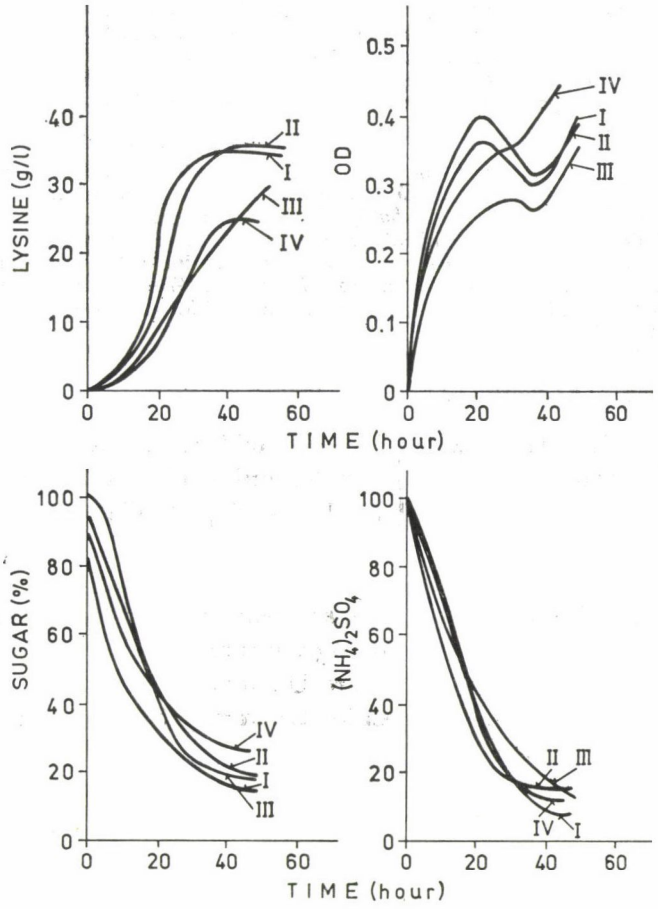

Fig. 4. Effect of different rates of aeration on lysine fermentation I = 0.6 l air l^{-1} min^{-1}; II = 1.0 l air l^{-1} min^{-1}; III = 1.4 l air l^{-1} min^{-1}; IV = 1.8 l air l^{-1} min^{-1}

Optimum values for lysine production were obtained at 15% sugar cane molasses concentration, at pH = 6.8–7.3 and aeration rate = 0.8 l air l^{-1} min^{-1}. Under these conditions cell metabolism was oriented towards the active synthesis of lysine at the expense of structural metabolism.

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NEW TECHNIQUE DEVELOPED TO ESTABLISH THE STERILIZATION REQUIREMENT IN THE CANNING INDUSTRY AND ITS APPLICATION IN PRACTICE

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By improvement of known analytical and evaluation methods and by the determination of new basic data a method was developed for the microbiological determination of an equivalent, which is characteristic of the true heat treatment requirement of product sterilization. The new equivalent, marked A, is of similar dimensions as the generally known F_0 value, however, it is calculated by a temperature constant (z) differing from that used for F_0 and by different basic data:

While for F_0 (min) $t_0 = 121.11$ °C and $z = 10$ °C, for A (min) $t_0 = 100$ °C and z: a specific value determined by measurement or experiment for the given product or group of products.

Thus, equivalent A is calculated on the basis of the heat destruction parameters, determined analytically, for the spoilage bacteria actually present in the product and not on parameters of selected identified or even fictive bacterial spores.

Equivalent A is, therefore, a basic parameter for sterilization practice.

For the sterilization of some vegetable products of the canning industry equivalent A calculated with $z = 20$ °C proved to be suitable. In the case of peas with the aid of thermobacteriological phase tests the equivalent, used to determine the heat treatment requirement, is calculated in accordance with the actual microbiological state of the product. With other canned vegetables, too, equivalent A calculated with the new basic parameter, $z = 20$ °C, proved to be suitable for the establishment of the sterilization requirement.

Due to the simple technique and the relatively non-elaborate equipment needed, the method may be applied to calculate the heat treatment requirement of other products of the canning industry.

The products of the canning industry are heat treated in up-to-date automated or even programmed equipment. However, a condition of the utilization of the possibilities of modern technic is the scientifically established sterilization* program but in this respect much is to be done yet.

The basic principles of thermobacteriology were developed in the 1920's by authors considered to-day the classics of this discipline (BIGELOW, 1921; ESTY & MEYER, 1922; BALL, 1923). Around 1960 a number of technical literature becomes available on the basic principles of the measurement and evaluation of sterilization and describes valuable data and experience (FLAUMENBAUM, 1960; HERSOM & HULLAND, 1963; HERRMANN, 1963; VAS, 1963; TELEGDY KOVÁTS *et al.*, 1963; CHEFTEL & THOMAS, 1963; STUMBO, 1965; NEHRING & KRAUSE, 1969).

* Sterilization is here understood to mean an operation suitable to produce irrespective of temperature and equipment "products of a long shelf-life at environmental temperatures".

The logarithmic mathematical model of thermal destruction of microorganisms in aqueous medium is considered an acceptable approximation. The equation of thermal destruction rate may be deduced from this model. Integration of the equation of velocity leads to the heat treatment equivalent which shows how many times the logarithmic destruction of the given (or presumed) bacteria at a given temperature in unit time (min) is increased. Accordingly, the equivalent may be expressed in units of time and is always related to a certain temperature. An important factor of equivalent calculation is value z and is understood to mean the temperature difference causing a tenfold increase in the rate of thermal death.

The oldest equivalent used in calculating the sterilization requirement was a round value, deduced from the investigations of ESTY and MEYER (1922) and relating to the reliable destruction of *C. botulinum:* $F_0 = 2.5$. Here the reference temperature is 250 °F = 121.11 °C and the temperature constant, $z = 18$ °F = 10 °C. A great number of equivalents and basic data determined by microbiological methods proposed for establishing the sterilization heat requirement, are available in the literature. Some of them, without laying claim to completeness, are as follows: STUMBO (1948); ALSTRAND & ECKLUND (1952); CHARM (1963); LANGE (1968); NATIONAL CANNERS ASSOCIATION (1968); NORMAN & DESROSIER (1970); FARKAS (1976b). The literature on analytical methods and methods of measurement is also plentiful, however, perhaps it is not necessary to go into it here.

The thermal death characteristics and the sterilization equivalents, however, deserve special attention. To characterize thermal death the following expressions are used: Decimal reduction time (DRT) or the value D which depends closely on temperature. This dependence is expressed in constant z as mentioned above. The thermal death time (TDT) is the multiple of D, most frequently $12 \times D$, however, other factors are also used. The process of thermal death depends also on the characteristics of the medium, thus, these have to be given, too: pH, water activity, *etc.* When the characteristics of thermal death are listed usually not only the species is specified, but the origin of the identified strain and the preparation operation are also described. In practice most useful are the data which were determined in products accounted for or in identical media.

The sterilization equivalent is calculated, by integration, from the thermal history determined by the measurement of temperature in the "cold point" or core. In the description of methods of calculations various z values are taken into account (STUMBO, 1965). Usually, however, an F_0 value, calculated with $z = 10$ °C is used for sterilization equivalent of products, even if the aim of sterilization is the destruction not only of *C. botulinum.*

Between the two fields of information, however, the correlation is not free of uncertainty. Sterilization processes based on practical experience,

lacking every scientific basis, are still encountered. Often the analysis of sterilization processes applied for several years and the calculation of the equivalents is given as fresh information without comparable, available microbiological data (TAKÁCS *et al.*, 1969; KÖLLER, 1969; EISNER, 1971, 1972; PAULUS, 1972; FLAUMENBAUM *et al.*, 1974a; REICHERT, 1974; ZÁHONYI, 1974; REICHERT & BIELIG, 1975; SEBŐK, 1975). In some of the sterilization programs an equivalent is not even mentioned (VAKÁNY & ÓR, 1976) and the equivalent determined by the measurement of operations is taken for granted, even if the process has been changed (FLAUMENBAUM, 1965; STUMBO, 1966; FLAUMENBAUM *et al.*, 1974b; KIZILOVA *et al.*, 1974).

The condition of a scientifically based technological determination of the heat treatment requirement would be the knowledge of the microbiological parameter needed to be taken into account in sterilizing a certain product. The problem is not only to select the appropriate microorganism the thermal death parameters of which serve as basic data in the given medium, but also how many times D thermal death has to be achieved. The solution may be derived from the determination of the thermal death characteristics of the characteristic spoilage bacteria in the given product (CASOLARI & GIANNONE, 1967; CZEGKA, 1967; GEORGESCU & BUGULESCU, 1969), of their initial density (SCHMIDT-LORENZ, 1959; BOLDOG *et al.*, 1975; SZÉKELY *et al.*, 1975) and of the tolerated residual cell count (PROSZT & VAS, 1960; FLAUMENBAUM, 1969). The residual cell count is understood to be specific for each product or group of products in view of the microorganisms affecting the keeping quality of the product (TAKÁCS, 1975). The experimental direct determination of the equivalent would be substantial simplification. Such an experimental result, however, may differ surprisingly from practical experience (SEEGER, 1968) and supports the opinion that the products are over-sterilized by the manufacturers, whereas it would be advisable to optimize the operations (HERRMANN, 1969; CZUKOR & ACZÉL, 1969; REICHERT, 1977). Probably the use of the available concrete, practical informations is encumbered by uncertainties (FARKAS, 1976a) still existing, inspite of the heartening increase of our knowledge as regards the mechanism of thermal death and of other environmental factors (FARKAS, 1969 and 1971; FARKAS *et al.*, 1975; FARKAS, 1976b).

Sterilization by heat is a thermotechnological operation defined by microbiology. It appeared, therefore, expedient to determine the parameters of the thermal death function interpreted as a mathematical model and thus attempt the dimensioning of the operation. The operation was first worked out for the sterilization of peas (CZEGKA, 1967 and 1968a and 1968b). Based on the results, publicizing of the method and its practical application was assisted by the RESEARCH INSTITUTE FOR THE CANNING AND PAPRIKA INDUSTRIES, Budapest (FÁBRI & CZEGKA, 1969; RESEARCH INSTITUTE FOR THE CANNING AND PAPRIKA INDUSTRIES, 1971). The CANNING FACTORY, Nagyatád, FRUIT

AND VEGETABLE CANNING FACTORY, Budapest and CANNING FACTORY, Szeged, made available their laboratories for testing the new method.

The basic principle of the new procedure, already proven by results is that a thermal death function is established characterizing the effect of the sterilization operation, based on the direct analysis of the actual microbial population present in the product. The practical application of the procedure was greatly assisted by the realization of a temperature constant (value z) substantially differing from the value hitherto generally accepted and by its determination (CZEGKA, 1970). It is interesting to note that BUGULESCU and co-workers (1968) calculate with a value z near to 20 °C, while FLAUMENBAUM (1965) calculates the equivalent with 15 °C.

The thermobacteriological dimensioning procedure, used throughout 10 years in the sterilization of canned peas was evaluated in detail and general conclusions were drawn in the thesis of the author of this paper (CZEGKA, 1977). The present paper gives an account of the results achieved in the thermobacteriological dimensioning of the sterilization requirement of peas and some other vegetables.

1. Principle and methods of the dimensioning procedure

In the mathematical presentation of the procedure the following data and symbols were used:

N logarithms of the viable cell count of microorganisms present in 1 g of the product to be tested. Culturing is used in the procedure, however, physical or biochemical selection is applied, thus, the result does not pertain to the total population.

N_0 is the initial value of N and is not directly determined during the procedure, it is generally calculated.

x time period (min).

t temperature (°C), t_0 = conventional comparative temperature.

D_0 Decimal reduction time at temperature t_0 (min).

z the temperature difference (°C) causing a ten-time change in the value D, describing the temperature dependence of thermal death, a constant characteristic of the microorganisms (temperature constant).

A_M equivalent as determined by microbiological test, calculated from the determined values of N_0, D_0, and z, basic data of dimensioning (min) interpreted as heat treatment time at t_0.

r tolerated spoilage by survival as percentage of the canned batch. Taken into account in calculating A_M.

G net unit canned product (g), taken into account in calculating A_M.

A_T technological equivalent calculated on the basis of the thermal history of the sterilization operation ($t = f(x)$) interpreted as the heat treatment

period at t_0 resulting in thermal death identical to that caused by the operation (min). Directly comparable to equivalent A_M.

The principle of the dimensioning procedure is summarized in equations 1, 2 and 3 and represented in Fig. 1.

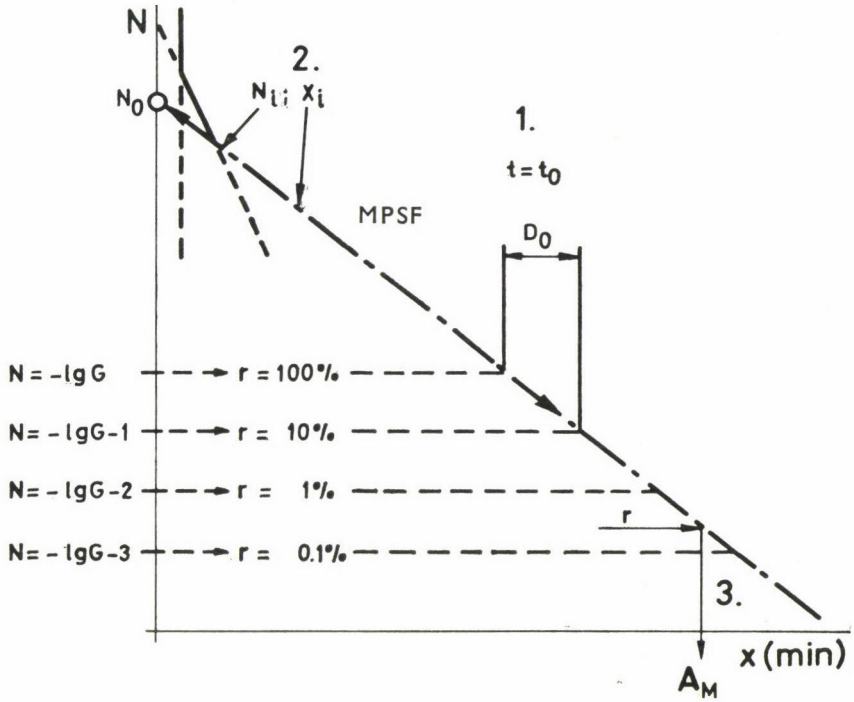

Fig. 1. Illustration of the principle of dimensioning procedure. Steps of the procedure: 1. determination of basic data D_0 and z; 2. on-line thermobacteriological test in order to determine N_i cell count of MPSF ordered to x_i treatment period at t_0 temperature as applied during the test; 3. calculation of equivalent A_M based on the thermal death function of MPSF, taking into account the tolerated spoilage by survivors r. Nomenclature: N logarithm of cell count, variable of the function; N_0 calculated value related to MPSF obtained in the course of the determination of N_i; t–t_0 temperature used for heat treatment and accounted for in calculating; x heat treatment period applied in the test, at the same time unit of measure of equivalent A_M; *MPSF* most probably surviving fraction, its thermal death function is illustrated by a dotted line; r percentage of spoilage by survivors; G net mass of the preserved units accounted for

Equation 1 is the thermal death function:

$$-\frac{dN}{dx} D_0 = 10^{\frac{t-t_0}{z}} \tag{1}$$

It describes the logarithm of thermal death in the given medium of a given form of a given species (*e.g.* spore) – hereinafter a given fraction. For each

fraction of the mixed microbial population as occurring in nature or in the products (multifraction systems) equations based on different D_0, z, N_0 parameters describe the change with time in cell count (reduction). In consequence of thermal death the proportion of cell counts between fractions is modified (Fig. 1). In the course of the process one fraction, the most probably surviving fraction (MPSF) remains only and represents by itself the cell count of the product. Presumably a natural population contains more fractions than it is shown in Fig. 1, and not only one but several of them may be accounted for as surviving fractions depending on their initial cell count, death characteristics or even on the effect of treatment. This possibility is rendered perceptible by use of the attribute "most probable".

The direct aim of heat treatment is the elimination of spoilage microorganisms. The composition of the product and the storage conditions to be expected determine which of the microbial fractions should be considered responsible for spoilage. In order to dimension heat treatment the MPSF of these fractions has to be accounted for. Determination of the spoilage causing fraction occurs by two-directional selection utilizing the appropriate medium and incubation as well as heat treatment.

A_M microbiological equivalent:

$$A_M = (N_0 - \lg r - 2 - \lg G)D_0 \tag{2}$$

is derived from the results of thermobacteriological investigations on the basis of the following presumption: the interpretation of the dilution cell counting technique is extended over the volume of manufactured batches. Accordingly, a single surviving microorganism may cause the spoilage of a single piece of product and thus r survivor per 100 canned product may cause the spoilage of at most $r(\%)$. In the knowledge of the mass of a unit the logarithm of the cell count causing $r(\%)$ spoilage may be defined on the basis of the above principle:

$$N = \lg r - 2 - \lg G$$

or in other words r number of cells in a mass of 100 G. The value of tolerated survival cannot be taken for 0 % because thus the value of N would be infinitely small, impossible to calculate with.

An N value obtained by calculating with an arbitrarily – *e.g.* on economic considerations – selected "tolerated $r(\%)$" as a tolerated residual cell count leads us, as illustrated in Fig. 1, to equation 2. Equivalent A_M means the heat treatment period needed to reduce at t_0 temperature, the value of the logarithm of cell count N_0 to N, as shown above.

Since the distribution of surviving cells (spores) in the product is most probably not even, equivalent A_M, as a basic data of technological dimensioning contains a high degree of safety.

Technological equivalent A_T is defined by Equation 3:

$$A_T = \int_0^x 10^{\frac{t-t_0}{z}} dx. \tag{3}$$

This may be calculated from the thermal history as established from the core temperature* measured during the sterilization operation by an adequate technique, probably by the help given in the appendix. Depending on the reproducibility of the operation and measurement, a more or less accurate mean value for $\bar{A}_T$ can be calculated from the repeated measurements.

At the same time, because of the stochastic character of the variables and parameters of the thermal death function of MPSF the statistical estimate of A_M can be established only from a satisfactory number of data. Thus, the two estimations: $\bar{A}_M$ and $\bar{A}_T$, are compared by an appropriate statistical method, *i.e.* by *Student*'s t test. As it may be seen, the dimension of the two equivalents is the same, while their origin and determination is different and independent from one another. Their comparison is the essence of the principle and practice of dimensioning.

It should be noted that it is expedient to choose identical t_0 temperature as base of comparison and as temperature applied in the investigation for the determination of A_M equivalent and D_0 decimal reduction time. In this case equivalent A_M is a number independent of the temperature dependence of death rate (z value).

The conclusion drawn from comparison of the equivalents remains a theory, only, as long as it is not verified by the satisfactory accord of the value taken into account in dimensioning and the actually observed spoilage percentage caused by survivors. Spoilage by survivors is tested by observation during storage in thermostat.

The conclusion drawn from the comparison of equivalents and spoilage is unambiguous and free of contradictions, if the temperature constant (z value) is suitably selected. It is essential, therefore, to try to determine and control the latter as will be explained later. That is why instead of F_0 equivalent calculated with a z value of 10 °C the A symbol is used for the equivalent derived from the thermal destruction function of product specific MPSF.

* The heat load, heat effect would be truly characterized by the thermal history of the complete mass of contents, which, particularly in the case of heat conducting products, is not identical with the thermal history of the core. With the products studied, readily mixed and characterized by heat convection, this problem did not arise.

1.1. Test by periodical heat treatment

This is a method suitable for use in on-line phase tests. It is indicated by its function that the product itself or its physically and chemically identical model have to be used as heat treatment and nutrient medium. The aim of this test is the selective determination of the spoilage causing MPSF.

At predetermined intervals sample is taken from the medium to determine, by culturing, the cell count during heat treatment in the laboratory at constant temperature. It is expedient to treat the product itself in sterile equipment after sterile preparation. The portions removed for culturing are injected into a suitable medium in a dilution series. After culturing the cell count is determined by one of the known methods. The N; x pairs of data, characteristic of the thermal death function of MPSF are achieved only after a certain heat treatment period, as shown in Fig. 1. By carrying out this test repeatedly from identical sample at different temperatures the thermal destruction function of MPSF may be determined in a multifraction system, *e.g.* in the product to be analysed. The function thus determined is valid only for the sample of which portions are taken but will be determined with values N_0, D_0 and z as well. By periodical heat treatment test of isolated MPSF strains in sufficient repetitions the values D_0 and z of individual strains may be reliably established. By repeated on-line sampling the frequency distribution or most probable value of the MPSF parameters (N_0, D_0, z) is also determined.

1.2. Thermobacteriological phase test

The result of the periodical heat treatment test of the sample taken from the manufacturing phase preceeding sterilization is equivalent A_M forming the basis for dimensioning. From the periodical heat treatment test at temperature t_0 the values D_0 and N_0 may be determined by evaluation according to Fig. 1 and equivalent A_M can also be calculated by Equation 2.

Dimensioning, naturally, is not prepared for a single case but for a technological procedure. The error inherent in the determinations (scatter) has to be taken into account as well as changes, fluctuations in the microbiological condition of production. Thus, dimensioning is based on the statistical estimation of A_M for a longer period. It is possible to supplement the test with further microbiological or other tests, measurements which permit the drawing of conclusions as to technological factors affecting the microbiological conditions.

If A_M changes in comparison to an earlier state by the sequential method then it is necessary to dimension a new sterilization technology by selecting another A_T equivalent level. In this case decision is made on careful deliberation of auxiliary tests and other information.

1.3. Representative constants

The statistical evaluation of the parameters of the thermal destruction function are affected to various extent, by random errors of measurement, however, their natural variability is also different. The cell count of MPSF (N_0) as the immediate characteristic of the microbiological condition of production, may change even day by day with daily conditions. However, the quality characteristics D_0 and z of MPSF were found practically non-changing or at most varying around their mean for a given product. Random errors, on the other hand, affect N one times, D_0 and z several time. Therefore it is expedient to use as D_0 and z parameters of MPSF representative values characteristic of the product. These are determined and continuously controlled by a satisfactory number of tests of the isolated MPSF strains, *i.e.* on the basis of a large number of data.

1.4. Series of experimental sterilization

Batches of the product under study are randomly taken from the production line and sterilized by processes of different thermal history. A part of the experimentally sterilized batches receives a treatment which permits substantial spoilage caused by surviving microorganisms during the storage test. The thermal history of the operations is recorded by measuring the core temperature and the A_T equivalent is calculated from these data.

Hereafter a correlation is sought for between the equivalent of operations and spoilage caused by survival. The conclusion is unambiguous only, or free of contradictions, if the representative constants, particularly value z, show a satisfactory agreement with the parameters of the thermal death function MPSF (all things considered, if the effect of sterilization and the underlying thermal death process may be truly characterized by the function correlation in question).

In view of the above the correlation as shown in Fig. 1 is not likely to be detected by regression analysis in such an experimental series. Because of the limited number of samples (a few hundred in ten–twenty-fold times repetitions) we are forced to coordinate the equivalents to a dual-chance quality characteristic instead of the measurable $r(\%)$, in other words: to "spoilage, or no spoilage". Thus, it has to be examined whether the equivalents ordered to one or the other event originate from two truly different realms of values (quasi of underlaying populations), whether spoilage is truly bound to lower equivalents. The result of this test permits of drawing conclusions as regards the correctness of value z taken into account in the calculations.

The method of experimental sterilization is suitable for the justification of equivalent A_M and representative constants D_0 and z, determined by phase test, being independent of laboratory analyses. It is, however, suitable also

for the selection of a value z accounted for in calculation and of the limit value A_T in default of thermobacteriological phase test method and of verified representative data. (In the majority of cases the latter conditions prevail since the complexity of thermobacteriological dimensioning was developed only for peas as yet.)

1.5. Application to canned peas

The thermal death characteristics (CZEGKA, 1967) determined for mesophilic spoilage bacteria in MPSF strains isolated in on-line tests, were used as representative constants:

$$D_0 = 20 \text{ min} \qquad (t_0 = 100 \text{ °C})$$
$$z = 20 \text{ °C}$$

The decimal reduction time advantageously measurable at 100 °C (not too short, neither too long) enabled the use of this temperature realizable by simple means as t_0 relative temperature. Thus, equivalent A_M calculated as the result of periodical heat treatment tests is independent of value z and is directly comparable to equivalent A_T calculated for $t_0 = 100$ °C relative temperature (CZEGKA, 1968a). For the calculation of the latter sterilization equivalent value $z = 20$ °C is used, as mentioned above. The thermobacteriological phase test developed on the basis of preliminary tests (CZEGKA, 1968b) was in the course of application improved (RESEARCH INSTITUTE OF THE CANNING AND PAPRIKA INDUSTRIES, 1971) and the technique thus developed is as follows:

The on-line phase samples are transferred to 300 cm³ sterile Erlenmeyer flasks or are prepared in them. To determine A_M the sample is taken in the phase immediately preceeding sterilization. The unit is thoroughly shaken and a sample, containing peas and juice in proportion, of about 100 cm³ is transferred to the sterile flask. As a complementing sample 50 g of precooked peas are taken directly with the sampling dish, not touching any other surface than the peas sampled; to this sample about 50 cm³ sterile 1 % brine is added to prepare it for subsequent heat treatment and inoculation in dilution series. The second complementary sample is taken from the brine under similar conditions prior to use, *i.e.* from the phase preceeding filling.

Samples are heat treated by direct boiling. It is desirable to reach boiling within 1–2 min and keep it boiling during 20 min. By immersing the sample into cold water it is cooled to the temperature where it can be inoculated.

Nutrient medium: the brine is diluted by double quantity of tap-water. To 1 l of this solution 5 g peptone, 1.5–2 g meat broth (OXOID) and 25 cm³ aqueous bromocresol purple (of 3 g per 1 solution) are added. The pH is ad-

justed to the blue colour of the indicator with some Na_2HPO_4. It is then portioned out into test tubes, sterilized at 120 °C and incubated for 48 h prior to use (sterility test).

Five times 1 cm^3 of the complementary samples are inoculated in test tubes containing the same medium. A dilution series is prepared from the sample taken prior to sterilization (using 9 cm^3 sterile water) in 3 or 5 parallels. The inoculations are evaluated by the known method, after incubation for 48 h at 37 °C. The result of the evaluation is the logarithm of cell count: N. (In evaluating the complementary samples the marks "negative" or "++" may also be used.)

A_M is calculated by Equation 2 from the N value as determined above. Members of the sum:

$N_0 = N + 1$, where the heat treatment period: 20 min is identical with value D_0. Taking tolerated spoilage to be 0.1%,

$$\lg r = \lg 0.1 = -1$$

The mass of units (cans of type 1/1 and jars of type 5/4) is uniformly 870 g, thus:

$$\lg G = \lg 870 = 2.94$$

Of the parameters only the value N, determined by analysis, is variable. Using the constant values the following simple equation is obtained:

$$A_M = (N + 6.94)20$$

The mean values of A_M equivalents obtained by phase tests of daily, weekly and seasonal samples are compared to the mean value of A_T sterilization equivalent as calculated from several measurements. Requirement: the mean value of A_T should exceed the mean value of A_M at least by the significant difference (*Student*'s test). Experiences, as given below, have shown the use of the probability level of P = 1% desirable.

The results of the complementary samples permit of drawing conclusions as to the origin of possible increase of A_M, caused by heavier contamination.

The conclusions have to be cross checked with the results of batch-wise storage tests subsequent to manufacture, in other words it has to be seen, whether spoilage due to survival does not exceed the value r (%) taken into account in determining the sterilization requirement. The storage test means the observation of the samples kept for 7 days at 37 °C. In its evaluation spoilage due to faults in airtightness have to be carefully separated from those caused by survivors.

1.6. Application of the procedure to other products

Of products containing only vegetables – according to present experience – canned peas are solely suitable for determining the thermal death parameters of MPSF by heat treatment at 100 °C. (Vegetables studied: string beans, cawliflower, tomatoes and pickles.) It is partly due to this fact that a similar procedure to the above could not be developed for other vegetables so far.

The dimensioning procedure, however, is applicable without the phase test by using serial sterilization test, sterilization equivalents and by comparing the results of spoilage due to survival.

2. Experiences with the application of the procedure

2.1. Sweet peas

Production technology of peas in short: the shelled, graded and blanched peas are filled into cans or jars and filled up with a brine containing 1–2% salt and 1–3% sugar. The containers are sealed and sterilized at temperatures around 120 °C. The pH of the product is nearly neutral: 6–6.4, therefore it is a good medium for spore forming bacteria (family *Bacillaceae)*. The MPSF comes partly from the sugar used as additive (CZEGKA, 1968b). By the two latter properties canned peas differ from other vegetable products having a lower pH and not containing sugar.

The procedure, as described in Chapter 1, consisting of phase test and dimensioning as well as serial sterilization test, forms a complete circle. In the case of peas this circle could be connected almost without gaps. Thus the procedure and mainly its principle may be studied by a typical example.

In the complete circle, naturally, numerical accord is not expected, only a statistical accord. However, the conclusions are bi-directional: pertaining, on one hand, to the heat load in accordance with the given microbiological state and to the qualification and factors affecting changes of the microbiological state, on the other. It follows from the above that by the joint analysis of tests and measurements a specific case of operation dimensioning is realized.

2.1.1. Dimensioning based on on-line phase test. The results of on-line phase tests carried out to check the application of dimensioning are summarized in Table 1.

Columns 2–6 of the table contain the results of the thermobacteriological phase tests as described in para. 1.5 of Chapter 1 and summarized according to season. Of these mean value $\bar{A}_M$ is the basic data of dimensioning. The results of the complementary phase tests (column 2–3) are summed up in the percentages of spore counts higher than normal and not in the mean value. In properly conducted processing, namely, in most cases, the spore count

Table 1

Dimensioning based on on-line phase tests

Serial number (year)	1. Thermobacteriological phase tests					2. A_T equivalent of sterilization operations				3. Comparison of equivalents and spoilage by survivors (Difference: A_T–A_M; sign given)					
	Complementary phases		A_M dimensioning equivalent			1/1 can		5/4 jar		1/1 can			5/4 jar		
	e (%)	f (%)	n	mean	scatter	symbol	mean	symbol	mean	Difference	Lowest significant difference ($P = 1\%$)	r (%)	Difference	Lowest significant difference ($P = 1\%$)	r (%)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0	0	14	133	8	TO	149	T1	225	16	13.8	0.208	92	35.1	0.045
2	17	27	26	139	15					10	9.0*	0.190	86	36.6	0.080
3	6	59	92	156	15					–7	7.2**	0.488	69	34.7	0.080
						T1	230			74	50.1	0.364			
4	17	50	99	165	17					65	50.4	0.139	60	34.7	0.032
5	13	43	112	179	19					51	50.3	0.354	46	34.7	0.192
6	6	36	38	158	12					72	50.4	0.578	67	35.0	0.031
7	38	62	24	167	12					63	50.4	0.328	58	35.0	0.118
8	95	89	36	208	31					22	24.8**	0.588	17	22.5**	4.404
						T2	312	T2	331	104	97	0.248	123	94.5	0.121
9	83	75	11	230	?					?	?	0.829	?	?	0.059
10	8	46	18	158	23	T1	230	T1	225	72	43.0	0.278	67	38.0	0.278
11	22	10	38	161	22					69	41.0	0.172	64	36.0	0.065
12	3	7	59	147	29	OHS	166	OHS	173	21	18.6*	0.078	28	23.4*	0.150

Columns 2 and 3: percentage of result "N > 0.2"; e (%): blanched peas; f (%): brine; n: number of tests

* $P = 5\%$;
** $P = 10\%$

of the brine and the blanched peas (detected as shown above) does not exceed the order detectable by 5 times 1 cm^3 inoculation ($N \leq 0.2$). The frequency of higher spore counts, therefore, indicates the degree to which the desirable microbiological condition was realized and indicates also the origin of the higher contamination by spores.

The sterilization operation is experimentally selected on the basis of the comparison of A_T sterilization equivalent, determined by measurement and A_M. $\bar{A}_M$ is the mean value as mentioned above, calculated for each season (Table 1, column 5). In determining $\bar{A}_T$ the random errors of the realization and measurement of the operation, have to be taken into account. Thus $\bar{A}_T$ has to be calculated from an adequate number of measurements but need not be repeated in each season because it is independent of factors characteristic of season – *e.g.* weather, amount of raw material, *etc.* – it depends only on the sterilization specifications and the type of sterilization equipment.

Data of sterilization operations and results of measurements are shown in Table 2.

The specifications of sterilization operations, T_0 for unit 1/1 and T_1 for unit 5/4, were prepared prior to the application of thermobacteriological dimensioning, thus, they were inherited specifications. As it can be seen in Table 1 (columns 7–10) the first three seasons were started with these specifications.

The joint evaluation of tests and measurements is obtained by statistical comparison of mean values $\bar{A}_M$ and $\bar{A}_T$ (Table 1, columns 11–16). The value of $\bar{A}_T$ should be higher than that of $\bar{A}_M$. It is decided by *Student*'s t test whether the difference between the two mean values exceeds the least significant difference.

The $\bar{A}_T > \bar{A}_M$ conclusion is the more sure the lower the P error probability at which the difference $\bar{A}_T - \bar{A}_M$ exceeds the least significant difference. The P as applied in the decision may be established on the basis of experiences. This shall be discussed later.

It is expedient to carry out the comparison separately for units 1/1 and 5/4, independently from the fact that $\bar{A}_M$ relates to both units, while significant difference between the mean values $\bar{A}_T$ is observed only in seasons 1–3. This is not only expedient because thus two parallel conclusions are reached, but because of the evaluation of the storage tests, too.

Conclusions drawn from the comparison are finally compared to the result of the storage test (column 13 and 16). In evaluating the storage tests only those spoilages are considered to be due to survivors where faults of air-tightness cannot be detected. Ranking of the two units is not of identical safety. The surface of closure in the case of jars can be safely checked after opening, however, in the case of cans faults of seal may remain undiscovered, *e.g.* gap in the sealing compound, break in the seal. According to investigations

Table 2

Equivalents of pea sterilization operations determined by measurement

Symbol	Packed units	Technological specifications of sterilization operations			No. of measurements	Results of measurements, equivalents					
						A_T equivalent $z = 20$ °C $t_0 = 100$ °C		F_0 equivalent $z = 10$ °C $t_0 = 121.11$ °C		F_{100} equivalent $z = 10$ °C $t_0 = 100$ °C	
						mean	scatter ±	mean	scatter ±	mean	scatter ±
1	2	3			4	5	6	7	8	9	10
		Autoclave operations									
		Heating up period (min)	Keeping period (min)	Sterilization temperature (°C)							
T0	1/1 can	7	19	118	9	149	11	7.6	1.1	982	142
T1	1/1 can	7	20	120	14	230	50	19.5	6.9	2519	891
TI	5/4 jar	20	20	120	18	225	51	13.5	7.8	1744	1007
T2	1/1 can	7	22	122	6	312	59	25.4	6.8	3280	878
T2	5/4 jar	20	22	122	8	331	76	25.2	8.2	3255	1059
		HUNISTER technology chain velocity m min[1]									
OHS	1/1 can	3.655			5	166	5	20.2	1.1	2607	142
OHS	5/4 jar	2.693			3	172	7	16.4	2.0	2120	155

HUNISTER technology: transit path of the chain through "G" steam chamber 26 m, through "M" hot and "H" cooling chambers each 9.75 m. Temperature in the chambers:

Chamber	M1	M2	M3	M4	M5	M6	G	U	H6	H5	H4
Temperature, °C	75	85	100	110	118	122	132	95	70	60	50

Note: $F_{100} = 129.16\ F_0$ is convertible, thus, comparable to A_M equivalents in Table 1, presuming $z = 10$ °C

of the author the latter may amount to 0.1–0.2 %. In view of practical expediency $r(\%)$, as shown in the table and accounted for in analyses, contains spoilage by survivors due to minor operating troubles or operational defects. Thus, the reproducibility of operations is taken into account and dimensioning is applied to conditions found in practice and not idealized. In consequence dimensioning is considered acceptable in case of spoilage by survival of the order of 0.1 %.

Now let us consider the conclusions drawn for seasons and the measures based on them.

According to the data pertinent to seasons 1 and 2 sterilization of units 1/1 (T_0) corresponds to the requirements as established above. Sterilization of units 5/4 is, however, overdone (T_1), thus it seemed indicated to reduce the heat load. In season 3, however, the sterilization of the 1/1 unit had to be modified. Its equivalent had to be raised to the level of T1 treatment. This modification was indicated by the formation of A_M, but it was supported also by the increase in spoilage by survival (column 13). After the modification of the sterilization procedure this increase was eliminated. The change in the microbiological state, as seen in the result of the phase test in the brine (column 3) was the consequence of the rearrangement of the technological line, in the course of which the pipe line of the brine was lengthened. However, the effect of the new arrangement could be compensated by the modification of sterilization. Sterilization at the level of T1 gave satisfactory results till season 7.

Two difficult years followed. In season 8 the value of A_M equivalent was high from the beginning. The complementary phase tests pointed also to an unfavourable shift in the microbiological state. All this was probably due to the unfavourable weather and the piling up of the raw materials. Based on the conclusions a new sterilization technology was developed, justified by the storage test of the samples manufactured during the evaluation period. This may be seen in columns 13 and 16. (Evaluation of the phase test of equivalents A_M and the experiment took 3–4 days, the storage test 6–7 days.) By introducing the new sterilization operations, marked T2, further increase in spoilage was prevented. These forced measures had to be continued during season 9, which was very short and difficult, it did not even yield an evaluable series of data.

During seasons 10 and 11 piling up of raw materials did not occur, thus, in accord with the results of thermobacteriological phase tests, it was possible to return to the previous technology and apply technology T1.

Based on experiences gained in the course of time it was possible to achieve some improvement in the microbiological conditions and the reduction of the heat requirement of sterilization. The measure taken was the reduction of the transit period resulting in the reduction of A_M as seen in row 12 in Table 1. The hydrostatic sterilization equipment (HUNISTER) reached about

the same time the stage of industrial application (SCHMIED, 1972). Automated control of this equipment enabled the reduction of scatter of the sterilization equivalent. Thus, it became possible to bring nearer to one another equivalents A_M and A_T and reduce the heat load. The result of the storage test proved the correctness of the procedure (Table 1, row 12 and columns 13 and 16).

Inspection of data in Table 1 permits the selection of the probability level of error, $P = 5\%$ in the analysis of criterion $\bar{A}_T > \bar{A}_M$.

It may be concluded, therefore, that the dimensioning procedure evolved is suitable for practical use and effectual.

It is worth noting, however, the importance of the numerical value of the temperature constant, $z = 20$ °C. A_M equivalent, determined at t_0 temperature is independent of the numerical value of z, thus it may be compared to any sterilization equivalent calculated with any value z, but identical t_0 temperature. In Table 2 equivalents, marked F_{100}, calculated for $t_0 = 100$ °C and with $z = 10$ °C are found comparable to the equivalents A_M of thermobacteriological phase tests. On the basis of this comparison, however, every operation would seem overdone and the conclusion, proven by experience, that in certain cases the heat load has to be increased, could not have been drawn. Thus, the concrete determination of the temperature constant is a condition of the procedure, but the application of the procedure enables the checking of value z.

2.1.2. Experimental sterilization series. By the technique as described in para. 1.4 it was possible to carry out a serial sterilization experiment in order to study the sterilization of canned string bean and sweet peas. Essentially the experiments for both products and their evaluation is identical, they differ only from two points of view. In the case of peas the existence of a threshold value of A_T equivalent, around 150 min, could be expected. In experiments with equivalents exceeding this spoilage by survival is not observed, while in experiments with lower equivalents appears spoilage. In the case of string beans no such threshold value could be fixed. The more important difference was that for peas this was a check-up experiment of an existing procedure, while for beans it served basic data for the development of a dimensioning procedure.

The experiments were carried out in the 5th season in an autoclave. Each batch consisted of 340–360 units, thus the observable spoilage per experiment could amount to 0.3–100% (r). The temperature was maintained between 110 and 130 °C for 5 to 25 min in order to obtain both, in a lower and higher temperature range positive (showing spoilage in the storage test) and negative reaction (showing no spoilage in storage tests). Each experimental series was divided into two groups on the basis of this simple practical point of view. Thus from the equivalents from a single experimental series two statistical samples were obtained. Then the significance testing of the means permits

raising the question whether the values of the samples generate from identical basic populations or from two discernible incidence intervals and whether actually spoilage by survival goes with lower equivalents. Thus the statistical test shows whether the equivalent really characterizes the bactericidal (preserving) effect of the operation. Since either A or F_0 equivalent is used, on the basis of the experimental results it can be established which of the two equivalents is suitable in practice for the dimensioning of sterilization operations.

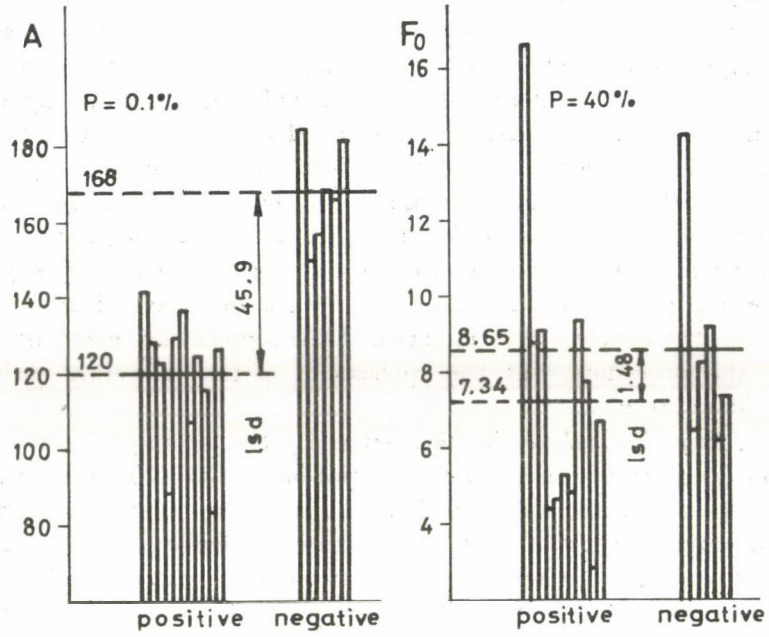

Fig. 2. Comparison of samples derived from the equivalents of serial sterilization experiments

Canned peas

Samples: *positive* sample – equivalents of experiments where spoilage by survivors occurred (inadequate effect); *negative* sample – equivalents of experiments where spoilage by survivors was not observed (adequate effect). The histograms represent equivalents A and F_0 of the experimental operations in the two parts in identical order. Horizontal lines stand for the mean values of the samples with numerical values. Vertical sections with arrows and numerical values show the *lsd* = lowest significant difference of the two means at the $P\%$ probability level as shown in the figure

Data of the experiments are shown in Figs. 2 and 3. As regards the statistical evaluation method the test is aimed at the so-called null hypothesis, according to which there is no difference between the mean values of the samples. Based on the statistical test the discarding of the null hypothesis justified the utility of the equivalent. This is the more safe the lower the probability level (P %) at which the null hypothesis is discarded. The equiv-

alents of individual experiments may be found in the figures (Fig. 2: 17 experiments with peas; Fig. 3: 18 experiments with beans). Naturally, for each experiment two equivalents are calculated, from the point of view of the test, however, it is no need to mark the related A and F_0 equivalents, because there is no correlation of the character of function relation between the two equivalents and comparison has to be carried out also one by one. The figures illustrate clearly the result of significance test. It may be seen in both experi-

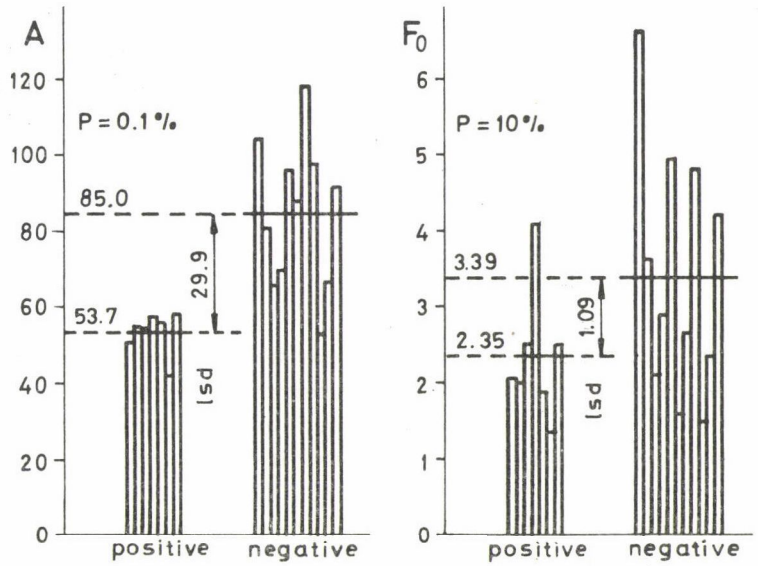

Fig. 3. Comparison of samples derived from the equivalents of serial sterilization experiments

String beans

Samples: *positive* sample – equivalents of experiments where spoilage by survivors was observed (inadequate effect); *negative* sample – equivalents of experiments where spoilage by survivors was not observed (adequate effect). The histograms represent equivalents A and F_0 of the experimental operations in identical order. Horizontal lines stand for the means of the samples with numerical values given. Arrowed vertical sections with numerical values given show the *lsd* = lowest significant difference of the two means at the $P\%$ probability level as shown in the figure

mental series that the A equivalents of the operations of inadequate effect, *i.e.* positive, are significantly lower than the A equivalents of effective operations (negative). In the case of peas the threshold value is at $A = 150$ min, as expected (Fig. 2). The threshold value for beans is found about 60–70 min. The F_0 equivalents of the samples derived from the experiments show practically no difference.

It is shown by the above that a relation exists between the keeping quality of the product and the A equivalent of the operation, while no such

relation exists between the keeping quality of the product and the F_0 equivalent of the sterilization operation. Thus, at least in the case of these two products, a temperature constant of the numerical value of 20 °C is expedient to be taken into account. F_0 equivalent is suitable only in judging the sanitary condition.

It is to be noted that the theoretically presumed quantitative correlation between spoilage by survivors as observed in the experiments and the sterilization equivalent could not be proven. (Pairs of data needed for the correlation calculations are derived only from the positive experiments.) Apart from this the results of the experiments are unambiguous, correspond to the basic principle of the dimensioning procedure, support the numerical value of $z = 20$ °C and for peas are in numerical accord with A equivalent determined in thermobacteriological tests.

2.2. Sterilization of some vegetables

Thermobacteriological test methods have to be developed for each product or group of products separately. In this difficulties are involved, thus it is not surprising that methods similar to the one applied to peas, are not available for most of the other products. In other words: data for MPSF of other products are unknown. Other canned vegetables, however, have been manufactured all along by technologies established on empirical basis. Thus, in the first step, the sterilization specifications traditionally applied, were studied. It was found that the specifications for the same product are often different. *E.g.* in the processing of peas the sterilization technology T0 was applied to 1/1 units, while T1 technology to 5/4 units, at the same time (Table 1). Comparison may lead directly to unification and in some cases this may result in the saving of superfluous heat load. On the other hand, ways of saving superfluous heat treatment may be sought by experimental series according to methods described in paras. 1.4 or 2.1.2 or even more simply. Thus, practical minimum equivalents may be established for individual products. A single equivalent can be established only in the knowledge of a reliable z value, utilized in the dimensioning procedure. In the case of uncertainity the desired safety may be achieved by the joint employment of two equivalents: A ($z = 20$ °C) and F_0 ($z = 10$ °C). It is, namely, rightly presumed that the $z = 20$ °C value is valid not only for MPSF found in peas and beans. The use of F_0 ($z = 10$ °C) equivalents, on the other hand, is indicated in view of prevention of food poisoning.

A frequent case of dimensioning tasks in practice is the modification of sterilization with the aim of improving quality, higher efficiency or the employment of a new technique. If the parameters for the MPSF of the given product, particularly the z value, is not available, at present in the majority of cases, dimensioning is best carried out on the basis of two equivalents.

Table 3

Recommended equivalents to the dimensioning of the sterilization of some canned vegetables

Product	A (min) $t_0 = 100$ °C $z = 20$ °C	F_0 (min) $t_0 = 121.11$ °C $z = 10$ °C
Peas		
in brine containing some salt and sugar pH: 6.0–6.4	150–300[a]	2.5[b]
String beans		
in brine containing some salt pH: 5.2–5.6	60–90	2.5[b]
with added acid		
pH: 4.5–4.8	60–70	2.0–2.5
pH: 3.8–4.2	40–60	0.2–2.0
Mixed vegetables		
peas, carrots, cawliflower in brine containing some salt pH: 5.4–5.6	90–100	2.5[b]
Cawliflower		
brine containing some salt and citric acid pH: 4.7–0.2	20–30	0.15–0.32
Tomato products		
purée, paprika in tomato juice pH: 4.2–4.5	2–10	0.003–0.01
Pickles		
Whole and cut cucumbers, paprika, etc. in brine containing acetic acid pH: 3.5–4.2	1.2–2.0	0.001

[a] Dimensioning according to the result of phase test
[b] Sanitary requirement minimum

In selecting the new procedure both equivalents should reach the equivalent of the earlier procedure, perhaps of proved value. Such equivalents along with the summary of the above are contained in Table 3.

3. Conclusions

The aim of this study was to develop a practical procedure for the thermobacteriological dimensioning of heat treatment. Practical and experimental experiences show that dimensioning of sterilization by heat in the canning industry may be solved on the basis of the mathematical model of the logarithm of thermal death, irrespective of the fact that the model from the scientific aspect, may be considered only an acceptable approximation.

The model is used in the procedure as the thermal death function of MPSF of the spoilage causing microorganisms. The constants of the function characteristic of the given MPSF, are D_0 and z values.

The cell count of MPSF can be determined by thermobacteriological tests of the samples taken from the line prior to heat treatment. From this the A equivalent, characterizing the heat treatment requirement, may be derived. Naturally, the MPSF cell count of the preserved product had to be mathematically expressed.

To characterize the bactericidal effect of heat treatment the equivalent derived from the thermal death function of MPSF is used as an index. This is calculated on the basis of the thermal history of the operation.

Practical experiences gained in the course of the study of pea processing proved the following:

1. By comparing A equivalent calculated on the basis of on-line thermobacteriological tests and of the thermal history of the sterilization operations the dimensioning procedure may be really and successfully applied.

2. The developed on-line thermobacteriological test permits the technological qualification of the microbiological condition, signalling its change and the determination of the necessary measures, *e.g.* the rational change of sterilization procedure.

3. It was proven in experiments that the A equivalent is a characteristic index of the bactericid effect of sterilization.

4. An important condition of the employment of the procedure is the determination of the actual value of z. In the case of MPSF in peas this value is 20 °C. This value, determined by thermobacteriological tests was supported by practical and experimental observations.

It was shown in an experimental series that the A equivalent calculated with $z = 20$ °C is suitable for the dimensioning of the sterilization of canned string beans, too.

The method of experimental series seems suitable for establishing value z (even for its determination) and the dimensioning of sterilization equivalents for products, where thermobacteriological test is not yet available. The safety of dimensioning may be increased by use beside of the generally applied F_0 ($z = 10$ °C) equivalent, equivalent A ($z = 20$ °C) as well as a dimensioning index with products, where data of MPSF are not determined yet.

It seems permissible to assign beyond the practical utility, theoretical significance to the thermal death function. However, the mechanism of thermal death and the explanation of the z values, 10 and 20 °C, were not investigated. The conclusions do not refer to F_0 equivalent used in judging wholesomeness.

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APPENDIX

Assistance to the calculation of the sterilization equivalent (Integration utilizing the trapezium formula). Thermal history is subdivided into sections of constant and steadily changing temperature. The initial temperature of each section t_i °C; time period: x_i. Solving equation 3 section by section:

$$\text{a)}\quad t = t_i \boxed{A_i = x_i h_i} \qquad h_i = 10^{\frac{t_i - t_e}{z}}$$

$$\text{b)}\quad t = t_i + \frac{t_{i+1} - t_i}{x_i}\, x \qquad \boxed{A_i = x_i \frac{j_{i+1} - j_i}{t_{i+1} - t_i}}$$

$$j_i = \frac{h_i z}{2.303}$$

Values are looked up in the tables as shown below:

$$t_i = 112\ ^\circ\text{C} \qquad h_i = 0.1227 \qquad j_i = 0.5328$$

Calculation of equivalent F_0; $t_0 = 121.11$ °C; $z = 10$ °C

j:

t °C	190	100	110	120	130	140		
0	0	0	0	3	3	6	2	0
1	0	0	0	4	2	3	2	5
2	0	0	0	5	3	2	8	5
3	0	0	0	6	7	0	8	1
4	0	0	0	8	4	4	5	0
5	0	0	1	0	6	3	2	
6	0	0	1	3	3	8	4	
7	0	0	1	6	8	5	0	
8	0	0	2	1	2	1	3	
9	0	0	2	6	7	0	6	

h:

t °C	90	100	110	120	130	140			
0	0	0	0	0	7	7	4	2	6
1	0	0	0	0	9	7	4	7	3
2	0	0	0	1	2	2	7	1	
3	0	0	0	1	5	4	4	8	
4	0	0	0	1	9	4	4	9	
5	0	0	0	2	4	4	8	5	
6	0	0	0	3	0	8	2	4	
7	0	0	0	3	8	8	0	6	
8	0	0	0	4	8	8	5	3	
9	0	0	0	6	1	5	0	1	

Calculation of equivalent A; $t_0 = 100$ °C; $z = 20$ °C

j:

t °C	60	80	100	120	140		
0	0	0	8	6	8	4	8
1	0	0	9	7	4	4	0
2	0	1	0	9	3	3	
3	0	1	2	2	6	7	
4	0	1	3	7	6	4	
5	0	1	5	4	4	3	
6	0	1	7	3	2	8	
7	0	1	9	4	4	2	
8	0	2	1	8	1	4	
9	0	2	4	4	7	6	

t °C	70	90	110	130	150		
0	0	2	7	4	6	3	
1	0	3	0	8	1	4	
2	0	3	4	5	7	3	
3	0	3	8	7	9	2	
4	0	4	3	5	2	5	
5	0	4	8	8	3	6	
6	0	5	4	7	9	5	
7	0	6	1	4	8	2	
8	0	6	8	9	8	3	
9	0	7	7	4	0	0	

h:

t °C	60	80	100	120	140		
0	0	0	1	0	0	0	0
1	0	0	1	1	2	2	0
2	0	0	1	2	8	8	9
3	0	0	1	4	1	2	6
4	0	0	1	5	8	4	9
5	0	0	1	7	7	8	3
6	0	0	1	9	9	5	3
7	0	0	2	2	3	8	7
8	0	0	2	5	1	1	9
9	0	0	2	8	1	8	4

t °C	70	90	110	130	150		
0	0	0	3	1	6	2	3
1	0	0	3	5	4	8	2
2	0	0	3	9	8	1	1
3	0	0	4	4	6	6	8
4	0	0	5	0	1	1	8
5	0	0	5	6	2	3	4
6	0	0	6	3	0	8	6
7	0	0	7	0	7	9	5
8	0	0	7	9	4	3	3
9	0	0	8	9	1	2	3

Equivalent of the procedure is obtained by adding up the equivalents of sections calculated one by one.

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STUDIES ON THE FACTORS AFFECTING GLUCONIC ACID AND 5-KETOGLUCONIC ACID FORMATION BY *ACETOBACTER*

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(Received 19 April 1979; accepted 7 July 1979)

Acetobacter suboxydans forms glucono-δ-lactone from glucose by direct oxidation. This is hydrolysed into gluconic acid, which in turn is oxidised into 5-ketogluconic acid. The results of this study have shown the ketogenic character of different *Acetobacter* strains to differ widely. In a highly ketogenic strain – *A. suboxydans* ATCC 621 – the effects of fermentation parameters upon growth, gluconic and 5-ketogluconic acid production were investigated.

Keto-oxidation was found to be substantially affected by the way of neutralization of the acid formed. If $CaCO_3$ is used for neutralization, it is sufficient to add one third of the theoretically required amount. This prevents the fermentation broth to become acidic to an extent detrimental to bacterial growth. By increasing the amount of $CaCO_3$ added, keto-oxidation is also intensified. By using Na_2CO_3 for neutralization, keto-oxidation is repressed due to the neutral pH. However, microbial growth is promoted by an acid medium (pH 4–5).

The percentage of 5-ketogluconic acid formed is not affected by the initial glucose concentration of the medium.

Investigation of the effect of temperature has shown the optimum growth to occur in the range of 297–299 K (24–26 °C) and the optimum rate of gluconic acid formation between 303 and 306 K (30–33 °C). In this range keto-oxidation is highly repressed. The difference in the temperature optima of product formation and of cell mass growth permits of the construction of the optimum temperature profile of gluconic acid fermentation with *A. suboxydans*.

On the basis of the present experiments the generally accepted kinetic pattern of gluconic acid fermentation has to be modified inasmuch as 5-ketogluconic acid fermentation is not a two-step process, *i.e.* 5-ketogluconic acid formation does not start after the total amount of glucose has been used up. Keto acid formation from gluconic acid, in accordance with the laws of consecutive biochemical reactions, begins in the initial phase of fermentation.

Description of detailed kinetic analysis of gluconic acid fermentation is to be found in the literature only in relation to moulds (PEPPLER, 1967; REHM, 1967) and to *Pseudomonas ovalis* (HUMPHREY & REILLY, 1965; KOGA *et al.*, 1967; TANNER, 1970). Relatively little is known of gluconic acid fermentation of the genus *Acetobacter*. Papers relate mostly to the biochemical characteristics of the strains (ASAI, 1968). As regards optimum gluconic acid formation by *Acetobacter suboxydans*, literature data are controversial. ASAI (1968) found the optimum pH at 4–6 and the temperature optimum at 298 K (25 °C). SOSHNIKOV (1970), on the other hand, found optimum product formation at 303–305 K (30–32 °C) and observed about 1% non-fermentable sugar in the final stage of fermentation.

5-ketogluconic acid formation by *A. suboxydans* was first described by *Kluyver* and *de Leeuw* in 1924. These authors obtained mostly qualitative

results. Further, the results obtained by other three authors deserve attention. In shaken culture experiments, KHESGHI (1954) found that, by increasing the glucose concentration, 5-ketogluconic acid conversion was repressed. YAMAZAKI (1957) found again in shaken culture experiments, the optimum of 5-ketogluconic acid formation to be at pH 4. Gluconic acid fermentation of *A. suboxydans* was perhaps most thoroughly examined by STUBBS and co-workers (1940) and the basic manuals quote also their investigations (AIBA *et al.*, 1965; RAINBOW & ROSE, 1963; *etc.*). They found that glucose fermentation in *A. suboxydans* occurs in two phases. Initially, in parallel to cell growth, glucose is converted into gluconic acid at an increasing rate and after the glucose had been used up the gluconic acid is converted into 5-ketogluconic acid. As regards temperature dependence of 5-ketoacid formation, they found 298 K (25 °C) more favourable than 303 K (30 °C).

In our days the biochemistry of gluconic and 5-ketoglunic acid formation of *A. suboxydans* is fairly elucidated (ÁSAI, 1968; SZŐKE, 1977). Glucose → gluconic acid conversion is catalyzed by two enzymes. One of the two is hexose oxidase independent of NAD (E. C. I. I. 3. 5. D-hexose: oxygen, oxidoreductase) bound to the cytoplasmic membrane with a pH optimum of 5.5. The other one is a soluble dehydrogenase with NAD or NADP co-enzyme (E. C. I. I. I. 47. beta-D-glucose: NAD(P), oxidoreductase) with a pH optimum of 8.6. As the result of the activity of these enzymes, first glucono-δ-lactone is formed from glucose which in turn decomposes spontaneously or by enzymic hydrolysis, into gluconic acid. The presence of lactanase enzyme in the cells was not unambiguously proven. In neutral or alkaline medium the glucono-δ-lactone → gluconic acid reaction shifts strongly in the direction of the acid. In the next oxidation step, as an effect of 5-ketogluconate reductase (E. C. I. I. I. 69. D-gluconate: NAD(P) oxidoreductase) gluconic acid is converted into 5-ketogluconic acid at a pH optimum of 7.5. The reaction equilibrium is highly disadvantageous for the 5-ketoacid. However, as an effect of $CaCO_3$ a significant amount of 5-ketogluconic acid is formed, because the reaction equilibrium shifts strongly in the direction of the insoluble Ca-5-ketogluconate. In addition to the above soluble 5-ketogluconate reductase, the presence of an NADP-independent 5-ketogluconate reductase enzyme bound to insoluble particles, was also proven. Its optimum pH was 4–6.

By the experiments carried out in the course of this study it was attempted to decide on, whether

– a difference exists in the ketogenic and the gluconic acid forming capacity of various strains of *Acetobacter* (called also *Gluconobacter*) *suboxydans* strains,

– outside factors (way of neutralization, pH, temperature, glucose concentration) affect and to what extent the glucose → glucono-δ-lactone → gluconic acid → 5-ketogluconic acid consecutive reaction series. Simulta-

neously it was attempted to find out which of the factors bear upon consecutive biochemical reactions most effectively.

1. Materials and methods

1.1. Strains used

Acetobacter suboxydans ATCC 621. American Type Culture Collection.

Gluconobacter suboxydans 3254
Gluconobacter suboxydans 3255
Gluconobacter suboxydans 3289
Gluconobacter suboxydans 3290
Gluconobacter suboxydans 3291

} Institute of Fermentation Osaka, Japan

1.2. Maintenance of strains

The strains were maintained in lyophilized state. Prior to each experiment the strains were cultured in Petri dishes on a medium of the following composition:

BACTO agar–agar	3.0%
BACTO pepton	0.5%
BACTO yeast extract	0.5%
Sorbitol	10.0%

After incubation for 48 h at 303 K (30 °C) an isolated colony was subcultured and again incubated. One colony was used to transfer onto test tubes. The subcultures in the test tubes were incubated for 48 h at 303 K (30 °C) and used as inoculum or stored for 1 week in the refrigerator at 281 K (4 °C). The medium in the test tubes was of the following composition:

BACTO agar–agar	3.0%
Corn-steep liquor	2.0%
BACTO yeast extract	0.5%
$CaCO_3$	1.0%
Sorbitol	10.0%

1.3. Preparation of the inoculum

The culture of a test tube was suspended in sterile water and used to inoculate two Erlenmeyer culture flasks of 750 cm^3 volume, containing 100 cm^3 sterile medium, each. Composition of the medium:

a. for batch fermentations:

glucose	10.0%
BACTO yeast extract	0.5%
$CaCO_3$	1.0%

b. in semi-continuous fermentations and in experiments utilizing Na_2CO_3 for neutralization (to avoid disturbance by $CaCO_3$):

sorbitol	10.0%
BACTO yeast extract	0.5%

Inoculum culturing was carried out under shaking at the rate of 250 strokes per min at 30 °C for 20 h.

For shaken cultures the inoculum was prepared in the same way and 5% w/w was used in 100 cm^3 medium in Erlenmeyer flasks of 750 cm^3. Cultivation was carried on for the required time at 30 °C. Composition of the medium in these experiments:

glucose	10.0%
BACTO yeast extract	0.5%
KH_2PO_4	0.5%
$CaCO_3$	1.0%

1.4. The fermentation process

Batch and semi-continuous fermentations were carried out in a *Biofer* fermentation system of 7 l useful volume, made in Hungary. During fermentation the parameters were kept at a constant level as follows:

a. in batch fermentations

agitation	600	rpm
air flow rate	0.5	$l^{-1}\,l^{-1}\,min^{-1}$
temperature	303	K (30 °C)

b. in semi-continuous fermentations

agitation	600	rpm
air flow rate	0.5	$l^{-1}\,l^{-1}\,min^{-1}$
pH	7.0 $\pm$ 0.2	

The fermentation medium contained 5–20% glucose and 0.5% BACTO yeast extract. To neutralize the acid formed, 1–2.6% $CaCO_3$ or 4 *N* Na_2CO_3 was used (the latter under automated pH control). The sterile fermentors were inoculated with 5% shaken culture. In semi-continuous fermentations, 25% of the previous fermentation was retained for inoculation.

1.5. Measurements

The cell density was characterized by the unit of optical density value (UOD). This was measured in a *Hilger* spectrophotometer at 600 nm. The glucose was determined by a glucose oxidase – catalase enzyme method (SUNDERMAN & SUNDERMAN, 1961). The reducing sugar content was estab-

lished by the method of *Schoorl* (BERNHAUER, 1939). The glucono-lactone was determined by LIEN's hydroxamate method (1959). The total amount of acid formed (gluconic + 5-ketogluconic acid) was calculated from the amount of Na_2CO_3 used for neutralization. Volume changes following from sample taking and the addition of Na_2CO_3 solution, were taken into account by corrections as given by LONGSWORTH and MACINNES (1936). Gluconic acid and 5-ketogluconic acid concentrations were obtained from the measured values by taking the difference of the above values. In the calculations, the lower molar reducing capacity of the ketoacid was accounted for (SZŐKE, 1977).

2. Results

2.1. Gluconic acid formation by Acetobacter suboxydans *strains*

The strains listed in Section 1.1 were compared in shaken cultures (Fig. 1). As regards cell growth, *Gluconobacter suboxydans* 3290 and *Acetobacter suboxydans* ATCC 621 proved to be the best strains. At the same time, as established from the residual reducing capacity, the ketogenic character of the strains differs substantially.

The same two strains were used in experiments carried out in laboratory fermentors. In these experiments (using $CaCO_3$ and Na_2CO_3, respectively, for neutralization) *Acetobacter suboxydans* ATCC 621 showed a stronger ketogenic character (Table 1).

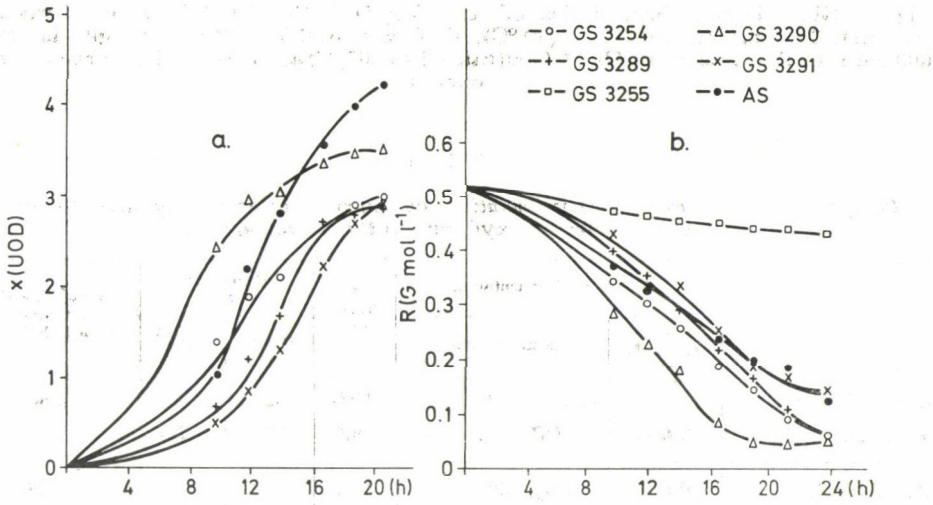

Fig. 1. Study of the shaken cultures of *Acetobacter (Gluconobacter) suboxydans* (GS, AS strains. [(rpm: 250, temperature: 303 K (30 °C), volume: 100 cm³ in an Erlenmeyer flask of 750 cm³] a. growth curves (X, cell density at 600 nm: UOD), b. curves of reducing capacity (R)

In further experiments, the strain *Acetobacter suboxydans* ATCC 621 was used since, in this strain, influencing the ketogenic properties could be expected to provoke better measurable, greater changes.

2.2 Effect of pH on gluconic acid formation

The acid formed during fermentation was first neutralized with $CaCO_3$. Figure 2 shows a fermentation where the added $CaCO_3$ was equivalent to about 36% of the acid formed. This amount proved to be sufficient to prevent the

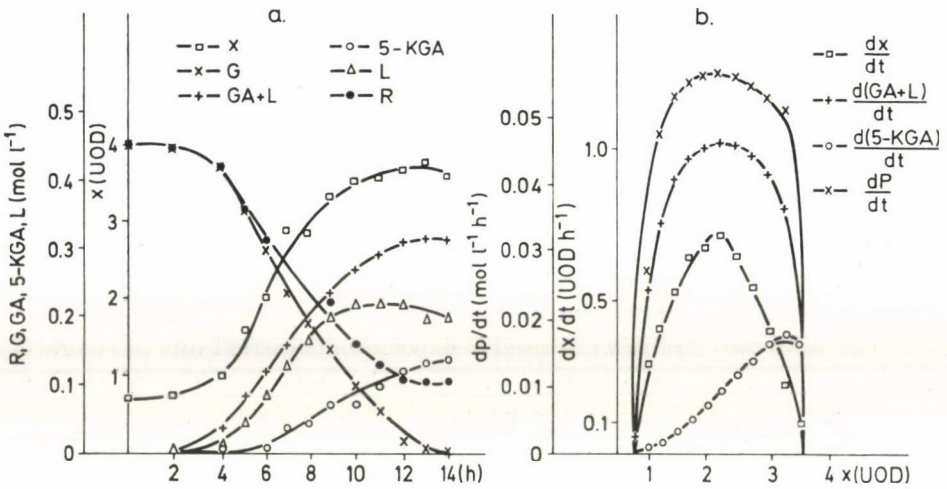

Fig. 2. Gluconic acid fermentation of *A. suboxydans* ATCC 621 in *Biofer* fermentor [volume: 7 l, temperature: 303 K (30 °C), air flow rate: 0.5 l l^{-1} min^{-1}, agitation rate: 600 rpm, in the presence of $CaCO_3$ (neutralized to 36%)]; a. concentration curves, b. rate curves

Table 1

Comparison of the gluconic acid fermentations by Gluconobacter suboxydans *3290 and* Acetobacter suboxydans *ATCC 621 strains, resp.*

Strain	Conditions of fermentation		* Time (h)	Compounds formed		Average rate of *P* formation (mol $l^{-1}h^{-1}$)10^2
	G_0 (mol l^{-1})	neutralization		GA (%)	5-KGA (%)	
Gluconobacter	0.455	$CaCO_3$, 36%	10.0	85.0	15.0	3.86
suboxydans 3290	0.650	Na_2CO_3, pH = 6	8.5	93.0	7.0	7.10
A. suboxydans	0.451	$CaCO_3$, 36%	13.4	70.0	30.0	2.36
ATCC 621	0.282	Na_2CO_3, pH = 6	9.0	81.6	18.4	2.56

* Fermentation period is understood to mean the time elapsing from inoculation to disappearance of glucose

pH of the fermentation broth to become so low as to inhibit the growth of bacteria after the glucose had been used up (at this stage pH 3.5). (Figs. 2 and 3).

In the fermentation shown in Fig. 3 100% of the theoretically expectable acid was neutralized with $CaCO_3$. It may be seen how strongly the fermentation was affected. The maximum bacterium density and the average rate of product formation is substantially lower than in the fermentation as shown in Fig. 2. At the same time keto-oxidation increased substantially. From the 10th h of fermentation, the quantity of gluconic acid diminishes.

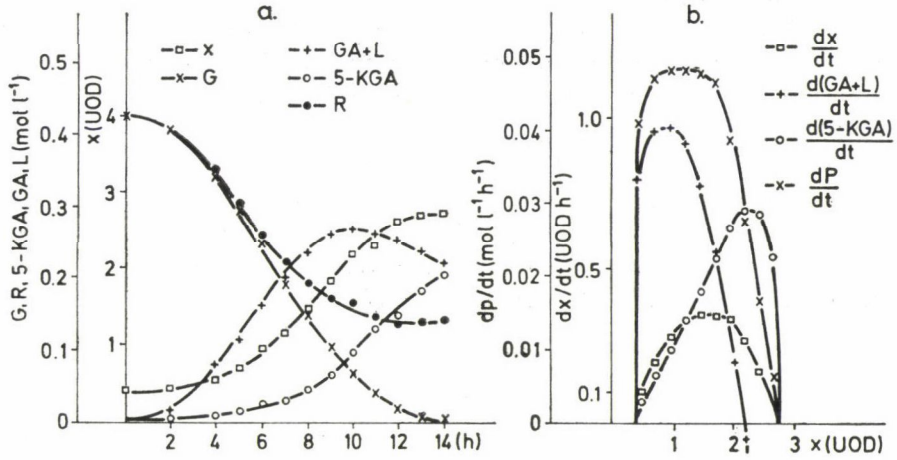

Fig. 3. Gluconic acid fermentation of *A. suboxydans* ATCC 621 in *Biofer* fermentor (for fermentation parameters see Fig. 2) in the presence of $CaCO_3$ (neutralization to 100%); a. concentration curves, b. rate curves

The main data on these two fermentations are summarized in Table 2. The Table contains the results of three parallel fermentations of the first type.

Table 2

Effect of $CaCO_3$ upon gluconic acid fermentation

No. of experiment	Neutralization with $CaCO_3$ as % of the total acid formed	Disappearance of glucose (h)	x_{max} (UOD)	GA formed as % of initial G	Initial glucose (mol l^{-1})	Average rate of P formation (mol $l^{-1}h^{-1})10^3$
1	36	13.4	3.80	70.0	0.451	2.36
2	36	14.0	4.10	63.6	0.542	2.46
3	36	13.3	3.40	71.6	0.469	2.53
Average	36	13.6	3.77	68.4		2.45
4	100	13.7	2.80	53.0	0.421	1.63

The difference between the two types of fermentations becomes apparent in cell growth and product formation curves as shown in Figs. 2 and 3. The difference between the $d(GA + L)dt$ curves is striking. In Experiment 4, the initial increase is followed by a rapid decrease, then its value becomes negative, *i.e.*, the rate of keto-oxidation is higher than that of GA formation. The maximum of curve d(5-KGA)dt is substantially higher than that shown in Fig. 2.

In further experiments the effect of neutralization with Na_2CO_3 was investigated. Fermentations were carried out in a pH-stat system. The width of the pH range applied was determined by the viability of the bacterium. Below pH 4 and above pH 7 the growth rate becomes very low. Therefore the experiments were carried out at pH 4, 5, 6 and 7. Table 3 contains data of these fermentations. The fermentation period is practically not influenced by the pH.

As seen in Table 3 and Fig. 4, the lactone accumulation is high at pH 4. At this pH L_{max} 40.3%, while at pH 7 it is 8.1%. In order to present this observation the cell growth and product formation curves obtained in fermentations, carried out at these two extreme pH values, are shown in Fig. 5 (Fermentation No. 6) and Fig. 6 (Fermentation No. 11), respectively.

Table 3

Influence of pH upon gluconic acid fermentation

No. of experiment	pH	Initial glucose concentration (mol l^{-1})	Disappearance of glucose (h)	x_{max} (UOD)	L_{max} as % of initial G	5-KGA as % of initial G	Specific glucose consumption rate ($G_{mol} h^{-1} x^{-1}_{max}$)
5	4	0.290	10.0	3.70	38.8	25.6	0.78
6		0.290	9.5	3.55	41.6	20.8	0.86
Average			9.75	3.63	40.3	27.2	0.82
7	5	0.306	8.0	3.65	32.4	22.2	1.05
8		0.288	9.5	3.35	38.6	23.6	0.90
Average			8.75	3.50	35.5	22.9	0.96
9	6	0.282	9.0	3.25	26.4	18.4	0.96
10	7	0.313	9.0	2.86	10.8	17.6	1.22
11		0.286	8.5	2.79	5.4	16.7	1.21
Average			8.75	2.83	8.1	17.2	1.215

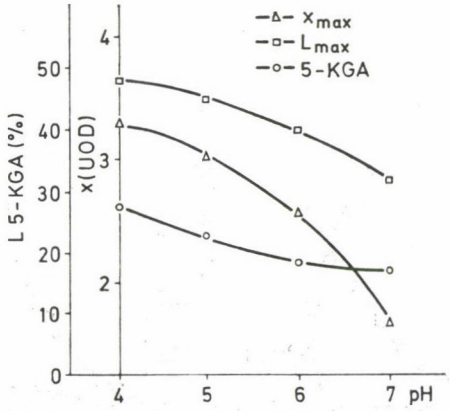

Fig. 4. Gluconic acid fermentation by *A. suboxydans* ATCC 621. Formation of maximum cell density (x_{max}), of maximum lactone concentration (L_{max}) and of final 5-ketogluconic acid concentration (for parameters see Fig. 2) at different pH using Na_2CO_3 for neutralization

The total product formation curve as calculated from the decrease of glucose at pH 7 (Fig. 6) is nearly identical with the total acid formation curve. Thus, lactone is present only in a very short phase of the fermentation and in a very small quantity. In Fig. 5 the two curves are rather different and lactone is present in the broth in a fairly high concentration. This can be explained by the dependence of lactone hydrolysis on pH. At higher pH values, the equilibrium shifts in the direction of the free acid. In this Figure the measured and calculated values of the amount of lactone were also compared.

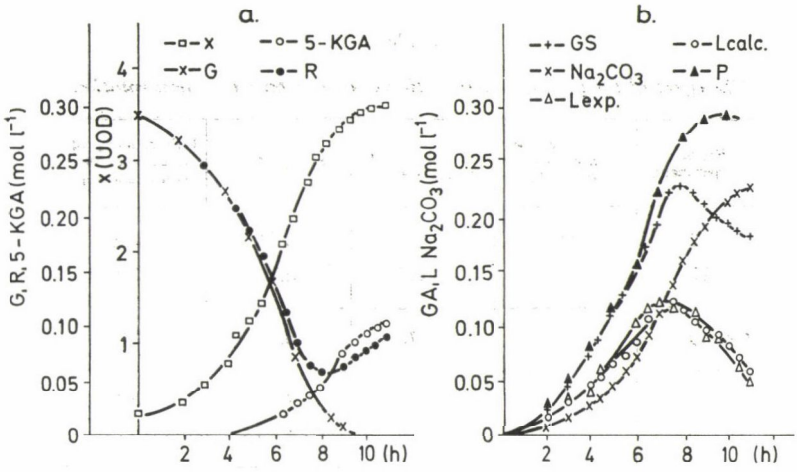

Fig. 5. Gluconic acid fementation by *A. suboxydans* ATCC 621 in *Biofer* fermentor (for parameters see Fig. 2) at pH 4 using Na_2CO_3 for neutralization

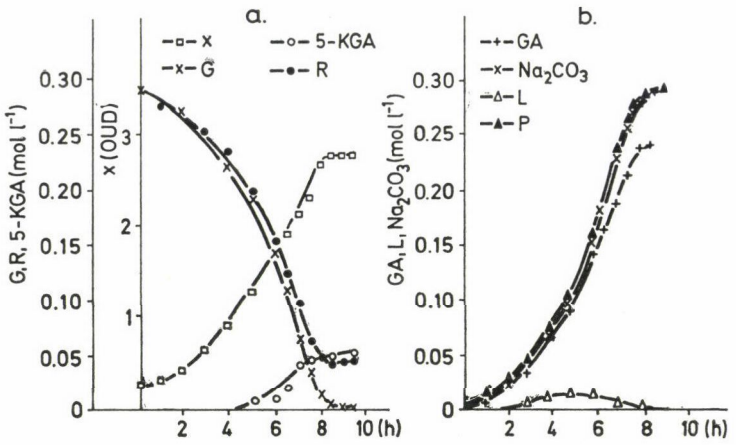

Fig. 6. Gluconic acid fermentation by *A. suboxydans* ATCC 621 in *Biofer* fermentor (for parameters see Fig. 2) at pH 7, using Na_2CO_3 for neutralization

A lower pH promotes 5-keto-oxidation, thus, from the point of view of gluconic acid formation higher pH is more favourable. Cell growth, on the other hand, is promoted by the lower pH, thus the pH optima of growth and product formation are not identical.

2.3. Effect of initial glucose concentration

The gluconic acid fermentation of *A. suboxydans* was studied at two different initial glucose concentrations. The pH level of the medium was kept constant at pH 5 by neutralizing the acid formed with Na_2CO_3. The main data of these fermentations are shown in Table 4.

Table 4

The effect of the glucose concentration upon gluconic acid fermentation

No. of experiment	Initial glucose concentration (mol l^{-1})	Disappearance of glucose (h)	x_{max} (UOD)	5-KGA as % of initial glucose concentration
12	0.28	8.0	3.65	22.2
13		9.5	3.35	23.6
Average		8.75	3.50	22.9
14	0.56	14.0	4.71	23.5
15		15.0	4.45	23.2
Average		14.5	4.58	23.35

The maximum bacterium concentration increased with increasing glucose concentration. Keto-oxidation was practically not affected by the initial glucose concentration, the percentage of the keto product did not change.

2.4 Effect of temperature

The temperature interval in which the experiments were to be carried out was set, on the basis of a preliminary study, between 294 and 309 K. Between these limits the temperature was raised by steps of 3 °C. These temperature differences are expected to affect the fermentation cycles to an extent permitting the demonstration of differences in the reaction rates far exceeding the margins of error of the methods used to follow the fermentations. The value of pH was kept at pH 7. According to previous experiments keto-oxidation is suppressed at this value and lactone accumulation is negligible. Thus, a direct information was obtained from the amount of alkali used under unit time as regards the rate of GA and 5-KGA formation. The endpoint marking the time for take off was, therefore, the end of alkali consumption.

The cultures needed to start semi-continuous fermentations were prepared by batch fermentation at 303 K (30 °C). Subsequent to this, the temperature dependence of semi-continuous fermentations was established in 5 (A/I) and 6 (A/II) runs carried out at different temperatures. In Experiment A/I, the temperatures applied were: 300, 297, 303, 306 and 309 K (27, 24, 30, 33 and 36 °C), in the above order. In Experiment A/II, the following temperatures were applied: 300, 297, 294, 303, 306 and 309 K (27, 24, 21, 30, 33 and 36 °C), in the order given.

The temperature sequence as given above was indicated by the fact that in periods of higher temperatures *A. suboxydans* degenerates, shown also by morphological changes. (Abnormal elongation of cells.) Results of experimental series A/II are shown in Fig. 7 and Table 5.

Table 5

Data of semi-continuous fermentation A/II gluconic acid fermentation as affected by temperature

Temperature		Fermentation period (h)	$x_{max}-x_0$ (UOD)	Quotient of $x_{max}-x_0$ and fermentation period	Initial glucose concentration (mol l^{-1}) 10^2	Formed (mol l^{-1}) 10^2		5-KGA (%)	$\left(\frac{\text{acid}}{x_{max}h}\right)$	$\left(\frac{x_{max}-x_0}{\text{acid}}\right)$
K	°C					Acid	5-KGA			
294	21	8.16	5.11	0.63	19.61	19.47	4.75	20.2	0.32	0.26
297	24	6.0	5.50	0.92	19.98	19.94	3.62	15.2	0.44	0.28
300	27	5.5	5.12	0.93	19.46	19.57	2.60	10.1	0.43	0.26
303	30	4.83	4.73	0.98	19.64	18.97	3.10	10.7	0.61	0.25
306	33	5.5	4.33	0.79	20.20	20.29	2.18	7.3	0.64	0.21
309	36	6.25	2.92	0.47	19.04	19.06	1.44	5.0	0.68	0.15

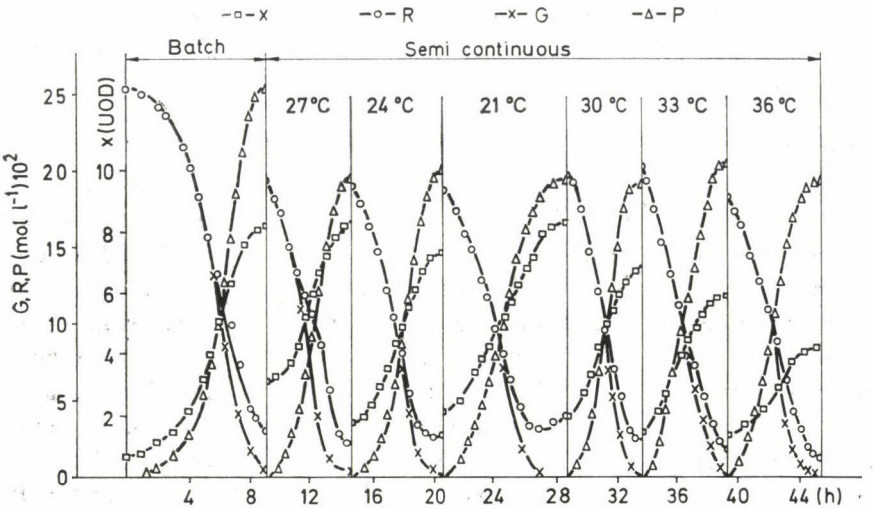

Fig. 7. Semi-continuous gluconic acid fermentation by *A. suboxydans* ATCC 621 in *Biofer* fermentor (for parameters see Fig. 2) with 25% inoculum at various temperatures

(As the results of series A/I were similar, the results are depicted in Figs. 8 and 9 only.) As seen in Table 5, the optimum glucose utilization rate (gluconic acid formation rate) was found at 303 K (30 °C). The optimum of 5-ketogluconic acid formation was found to be at the substantially lower temperature of 294 K (21 °C) and, by increasing the temperature, the percentage ratio of the keto product decreased from 20.2% to 5%. The percentage of 5-ketogluconic acid formed within one period as related to the total amount of acid formed by the end of the period, is shown in Fig. 9. Changes in x, indicative of cell growth during fermentations ($x_{max}-x_0$), as a function of temperature, are shown in Fig. 8.

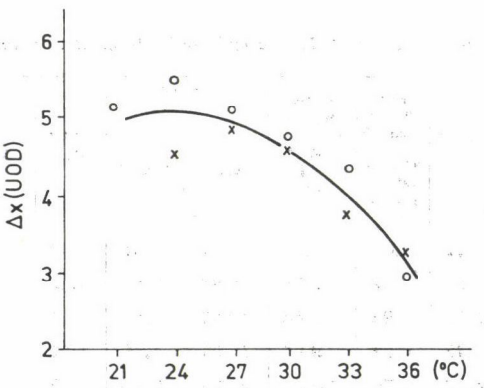

Fig. 8. Growth of *A. suboxydans* ATCC 621 as affected by temperature ($x = x_{max}-x_0$) in experimental series A/I and A/II (x and o) in *Biofer* fermentor (for parameters see Fig. 2)

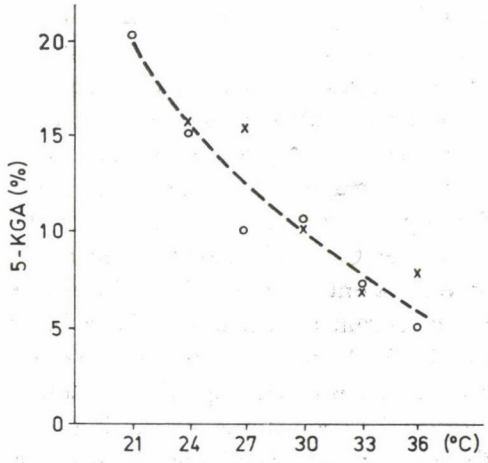

Fig. 9. 5-ketogluconic acid formation by *A. suboxydans* ATCC 621 as affected by temperature (5-KGA = ketoacid formed as % of total acid) in experimental series A/I (x) and A/II (o) in *Biofer* fermentor (for parameters see Fig. 2)

Lower temperatures favour cell mass formation although the optimum of the average growth rate is still at 303 K (30 °C). The value of acid/x_{max} · fermentation period representing specific acid formation rate increases with increasing temperature. The average specific acid formation rate doubles in the temperature range examined. The amount of acid formed in a temperature cycle related to the cell mass is relatively constant between 294 and 303 K (21–30 °C), however, at higher temperatures, it increases substantially.

3. Conclusions

A substantial difference was observed in the fermentation rate and ketogenic property of various *A. suboxydans* strains. The strains may be used for gluconic acid production where the small amount of 5-ketogluconic acid is not disturbing. According to the results obtained the formation of keto acid may be effectively repressed. The gluconic acid forming capacity of bacteria is substantially higher than that of slowly growing moulds (SZŐKE, 1977; RAINBOW & ROSE, 1963). The consecutive biochemical (microbiological) reaction selected in the present study, may be influenced by the following factors:

– the initial glucose concentration,
– way of neutralization,
– pH,
– temperature.

It was found in this study that, in contrast to data in the literature (KHESGHI, 1954) the proportion of gluconic to 5-ketogluconic acid (Table 4), was not affected by the initial glucose concentration. Productivity is increased by increasing the glucose concentration (the rate of glucose utilization increases from 3.2 to 3.86 · 10^{-2} mol l^{-1} h^{-1}) while, because of the reduced overall yield constant, conversion increases also somewhat. Due to economic reasons these two factors definitely indicate the use of a higher glucose concentration.

It was found that when $CaCO_3$ is used for neutralization it is sufficient to apply the amount equivalent to one third of the theoretically expected acid. This is sufficient to prevent acidification of the fermentation broth, in a degree detrimental to microbial growth. The use of more $CaCO_3$ substantially increases keto-oxidation because of the equilibrium shifting effect of the insoluble Ca-5-ketoglunocate formed. If the production of 5-keto acid is aimed at, great amounts of $CaCO_3$ have to be applied. This observation is in accordance with the results obtained by STUBBS and co-workers (1940). The present experiments did not prove the finding of the above authors, namely, that gluconic acid fermentation by *A. suboxydans* is a two-step process, or in other words, that 5-ketogluconic acid formation begins only when the conversion of glucose to gluconic acid is finished. As can be seen in Figs. 2 and 3, ketoacid formation begins immediately after the appearance of gluconic acid and a greater amount of $CaCO_3$ shifts only the reaction equilibrium in the direction of 5-ketogluconic acid. It is evident that STUBBS and co-workers (1940) used a strain of very low ketogenic capacity and could not differentiate between gluconic acid and 5-ketogluconic acid. Thus, they made an error in extrapolating the glucose consumption and 5-ketogluconic acid formation curves (Fig. 10). Various authors (AIBA *et al.*, 1965; RAINBOW & ROSE, 1963; REHM, 1967) adapted this pattern of fermentation, without controlling it and mention it as a specific type of fermentation. However, this is in contradiction to the general kinetic law of consecutive reactions, according to which in reaction

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

$dc/dt = k \cdot B$, thus the formation rate of the final product is proportional with the concentration of the intermediate product. Using an analogue computer to model this system, a kinetic pattern identical with that obtained in the present study is found (RÖPKE & RIEMANN, 1969) (Fig. 11).

A conclusion, similarly erroneous as that of STUBBS and co-workers (1940) is drawn by SOSHNIKOV (1970) from his experiments, maintaining that at the end of gluconic acid fermentation in *A. suboxydans* about 1% of non-fermentable sugar remains. It is evident that this consists of 5-ketogluconic acid formed in the course of fermentation and not of residual glucose.

The experiments where Na_2CO_3 was used for neutralization and the pH was kept constant have shown that the time of fermentation is not affected

by the pH, but microbial growth is greatly affected. At a lower pH, the cell mass formed is larger, while this pH promotes 5-ketogluconic acid formation. This is in agreement with the finding of YAMAZAKI (1957) who found the optimum for keto acid formation to be at pH 4. Presumably the insoluble 5-ketogluconate reductase is located in the cytoplasmic membrane and the pH of

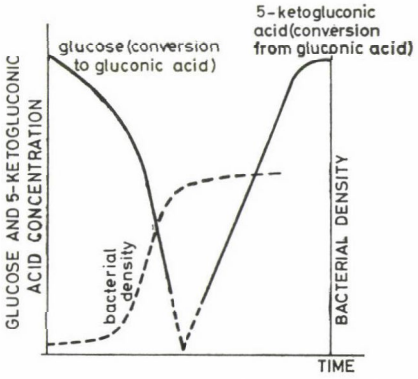

Fig. 10. Two-phase gluconic acid fermentation by an *A. suboxydans* strain (AIBA *et al.*, 1965)

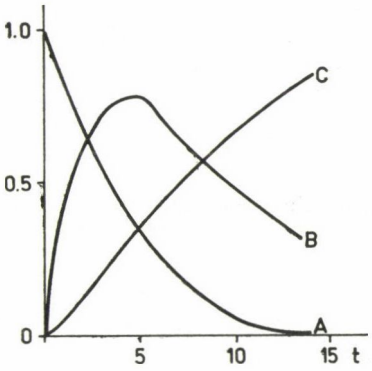

Fig. 11. $A \xrightarrow{k_1} B \xrightarrow{k_1} C$ consecutive reaction as simulated by an analog computer (RÖPKE & RIEMANN, 1969). Hypothesis: reaction of the first order, $k_1 = 0.5$, $k_2 = 0.1$

the environment is optimal for this enzyme. At the optimum pH of 5-ketogluconic acid formation the lactone concentration is high and in this compound the 5th carbon atom of the gluconic acid is bound to a lactone ring. This contradiction may be interpreted in two ways: either the affinity of 5-ketogluconate reductase is higher to lactone than to gluconic acid (which is not likely), or lactone, being an indifferent molecule, may pass the cytoplasmic membrane easier than gluconic acid. Thus the soluble 5-ketogluconic acid reductase enzyme in the membrane and within the cell is capable of converting

the gluconic acid formed spontaneously or by enzymatic hydrolysis in the slightly alkaline medium of the cell, into keto acid. This presumption served to construct the model of the gluconic acid forming mechanism as shown in Fig. 12 (HOLLÓ *et al.*, 1978).

According to Table 3, keto-oxidation is repressed in a neutral medium (pH 7), *i.e.*, this pH is favourable for gluconic acid formation. This finding is in partial contradiction to that of ASAI (1968) who found the optimum of gluconic acid fermentation to be at pH 4–6.

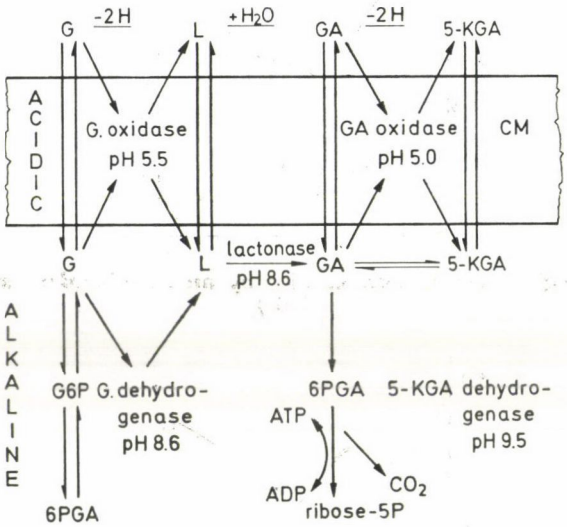

Fig. 12. Probable mechanism of glucose → glucono-δ-lactone → gluconic acid → 5-keto gluconic acid consecutive conversion (Model of hypothesis)

It may be seen from the above that the total acid formed by unit cell mass or the specific product formation rate is higher in neutral medium than in an acid medium. This further means that the conversion quotient and thus, also the efficiency of the process is higher at pH 7.

The effect of temperature was studied in semi-continuous fermentations. With increasing temperature the microbial concentration and the amount of ketogluconic acid formed decrease. This is in agreement with the observations of STUBBS and co-workers (1940) who found 298 K (25 °C) to be more favourable for ketogluconic acid formation than 303 K (30 °C). The difference in the optimum temperature of microbial growth and of gluconic acid formation permits the mathematical modelling of gluconic acid formation by *A. suboxydans* and, based on this, the construction of the optimum temperature profile of fermentation (SZŐKE *et al.*, 1974; NYESTE *et al.*, 1979).

It is interesting to note that even at a temperature distant from the optimum for growth, at 309 K (36 °C), a significant gluconic acid formation rate is found, though this temperature is responsible for a definite degeneration of bacteria. According to the Y_{ATP} conception (BAUCHOP & ELSDEN, 1960) a close correlation exists between the mechanism of energy production (here gluconic acid formation) and energy requiring cell biosynthesis (cell yield) in a growing culture. However, sometimes this correlation may be disrupted. Such disruption may be due to increased temperature (SENÉZ, 1962). Perhaps in this case, too, a distruption is responsible for the fact that microbial growth ($x_{max} - x_0/acid$) related to one mol acid formed (one mol glucose) is relatively constant within a wide temperature interval (see Table 5) but the quotient substantially decreases (from 0.26 to 0.15) at 309 K (36 °C). In consequence both the rate of product formation and the gluconic acid yield increases with increasing temperature (the microbe uses a lower quotient of glucose for assimilating cell formation at higher temperature).

The experiments have shown that the equilibrium conditions of the consecutive biochemical reaction studied may be influenced mainly by the temperature, by the way of neutralization (addition of $CaCO_3$) and the pH. Thus, in optimizing other biochemical consecutive reactions the above fermentation parameters have to be used in the research project.

Nomenclature

G	Glucose concentration (mol l^{-1})
GA	Gluconic acid concentration (ml l^{-1}) (g 100 ml^{-1})
5-KGA	5-ketogluconic acid concentration (mol l^{-1}) (g 100 ml^{-1})
x	cell density (UOD)
x_{max}	maximum cell density (UOD)
x_0	initial cell density (UOD)
L	lactone concentration (mol l^{-1}) (g 100 ml^{-1})
L_{max}	maximum lactone concentration (mol l^{-1}) (g 100 ml^{-1})
L_{exp}	lactone concentration measured (mol l^{-1})
L_{cal}	lactone concentration calculated – ($P - GA - 5$-KGA)
R	reducing sugar content (mol l^{-1} according to Schoorl)
P	amount of total product ($GA + L +$ 5-KGA)
CM	cytoplasmic membrane
6 PGA	6–phosphogluconic acid
$G6P$	glucose–6–phosphate

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INHIBITION OF RANCIDITY OF FATS BY PAPRIKA AND TOMATO SEEDS

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In order to retard rancidity of fats experiments were carried out with additives of natural antioxidant activity.

Of the additives tested, paprika seeds and tomato seeds, by-products of industrial processing, are discussed. The seeds were ground and added to various fats. Alternatively, the seeds were extracted with different solvents and added as isolates. The effect of these agents was established by comparing them with fatty substances free of additive or containing BHT as an antioxidant.

In order to accelerate oxidation, samples were placed into a drying oven or a thermostat, or the active oxygen method was applied. The antioxidant activity of seeds was tested also at room temperature. Advance of oxidation was followed up by peroxide value determinations.

The seeds, added at a concentration level of 5%, were found to ensure stability nearly equal to that provided by 0.01% BHT. Of the known antioxidants the seeds contained tocopherols, ascorbic acid, of the synergists citric acid, cephaline and lecithin were present. However, the extracted agents, when blended with fatty substances did not give the result expected.

The experiments are being continued to identify the active agents and to establish their effect.

Experiments hitherto carried out have shown paprika and tomato seeds to be suitable for retarding oxidation of the fats in animal feeds and premixes when directly admixed.

Inhibition of autoxidation in fats, fat-containing foodstuffs, fat-containing premixes and feeds is a central problem of antioxidant research. In order to retard oxidative spoilage, synthetic antioxidants are normally used. This is due to their simple and economical production and their utility in a wide range. To elucidate the health aspect of synthetic antioxidants, a thorough investigation was started in recent years. The toxicity of some synthetic antioxidants was discussed in numerous papers in the last 10 years. ICHIKAWA and co-workers (1971) report mainly on their research into phenolic type antioxidants (BHA, BHT). FURIA and BELLANCA (1976) treat the same subject. They found that a substantial amount of these substances is absorbed by various organs of the animals and the continued consumption is of toxic effect. Their findings support and complement the observations of earlier authors.

Research into antioxidants is aimed at reducing the use of synthetic agents or eliminating them. Of the natural antioxidants tocopherols, ascorbic acid and its esters, and their mixtures with various synergists are of highest interest (TELEGDY KOVÁTS *et al.*, 1969; BERNDORFER-KRASZNER, 1966; POKORNY, 1974; PONGRÁCZ, 1973; PERÉDI, 1973; BENDICT *et al.*, 1975; SEDLACEK, 1975; KLÄUTI, 1976). The antioxidant effect of flavones is reported

by SIMPSON and URI (1956), and GÁBOR (1976). KAWASHIMA and co-workers (1977) discuss the antioxidant activity of the combinations of carbonyl compounds and amino acids.

POKORNY and co-workers (1976) continue research on natural antioxidants by studying the phospholipids of soya, SEHER and IVANOV (1976) by investigating cumin oil and employing it as an antioxidant. HAMMER (1977) found antioxidant effects with cumin and coriander. KRIPAK and co-workers (1973) found the extracts of juniper and black pepper to be effective antioxidants. Of aromatic herbs the antioxidant activity of rosemary and sage was studied by CORT (1974), RUSSO (1976), SAITO and co-workers (1976a, 1976b) and many more authors.

The aim of this study was to support work of detecting native substances of antioxidant effect. Spices and their by-products, used in our everyday food, were studied. Rancidity tests were carried out with ground paprika, paprika seeds, and tomato seeds.

The present paper gives an account of experiments carried out with paprika and tomato seeds and with some of their components.

1. Materials and methods

The following fats and agents of antioxidant activity were used in the experiments:

Fatty substances

- Lard
- Sunflower seed oil
- Rape seed oil
- Trioleate (BDH, Chemicals Ltd., England)

Substances used as antioxidants

- Paprika seeds
- Tomato seeds
- BHT (Butylated-hydroxy-toluene)

1.1. Preparation of samples for experimental use

In order to study the antioxidant effect of paprika and tomato seeds, the ground seeds or their extracts (isolates) were used. The ground seeds and extracts were blended with the fat in the same way.

The substances employed as antioxidants were utilized in the following manner:

– Ground paprika or tomato seeds of a particle size of 100 mesh were blended at concentration levels of 1–10% (w/w) with the fat. After establishing the optimum concentration this value was used.

– Tocopherols were extracted from the paprika and tomato seeds, respectively (BERNDORFER-KRASZNER, 1970) and an amount of extract corresponding to 5–10% seed was blended with the fat.

– Ground paprika or tomato seeds were extracted in a *Soxhlet* apparatus during 4 h with *n*-hexane. After evaporating the solvent, the residual isolate – containing according to thin-layer chromatography tocopherols beside lipids – was blended with the fatty substance in a ratio corresponding to 5% seed (w/w).

– Ground tomato seeds were extracted in a 2 : 1 chloroform – methanol mixture for 24 h in a thermostat at 311 K (38 °C) according to FOLCH and co-workers (1957). The mixed solvent was evaporated under vacuum at 313 K (40 °C) and the residue was blended with the fatty substance in the above proportion.

– The mixture of lipids as obtained in the previous para. was separated into neutral lipids and phospholipids on a metasilicilic acid column (DUTTERA *et al.*, 1968). The two fractions were separately blended with the fatty substance.

– Ground tomato seeds (corresponding to 5% of fat) were extracted in solvents of increasing polarity, in succession. Each extraction lasted 4 h. The solvents were as follows: *n*-hexane, benzene, chloroform, methanol and finally distilled water. Each solvent was evaporated and the residue blended with fat.

– The seeds, after extraction according to the previous para. were blended with fat.

– Ground tomato seeds were extracted in a *Soxhlet* apparatus with a 1 : 2 : 1 mixture of water–hexane–ethanol for 4 h. The isolate was blended with fat at a ratio corresponding to 5% seed.

– BHT blended with fat served as the comparative sample. The BHT was dissolved and admixed in a proportion of 0.01%.

– Fat without additive was used as a control.

1.2. Methods of analysis

In order to accelerate oxidation, of the many methods suggested in the literature, placing in a drying oven, in a thermostat and the AOM (Active Oxygen Method) were applied. Further rancidity experiments were carried out at room temperature, in daylight.

The temperature of the drying oven was set at 373 K (100 °C), that of the thermostat at 323 K (50 °C). With the active oxygen method the temperature of the fat was 371.5 K (98.5 °C) and the rate of air flow through the fat 40 l min^{-1}.

Changes as induced by oxidation were checked by determining the peroxide value (HUNGARIAN STANDARD, 1973)

1.3. Mathematical statistical methods of evaluation

In order to evaluate results, standard deviation was calculated and analysis of variance was done based on the following relationships:

Standard deviation:

$$s = \sqrt{\frac{(x-\bar{x})^2}{n-1}}$$

where x are measured values
$\bar{x}$ mean value of data measured
n number of measurements

Coefficient of variation:

$$v\% = \frac{s}{\bar{x}} 100 .$$

Analysis of variance:

from the table: SQ sum of measured data
DF degree of freedom
MQ variance of error
F value of *Fischer*'s F test
*** significant at the probability level of P = 0.1%
** significant at the probability level of P = 1%
* significant at the probability level of P = 5%

Least significant difference:

$$SD_{5\%} = t_{5\%} \sqrt{\frac{2 \cdot MQ}{i}}$$

where $SD_{5\%}$ least significant difference at the probability level of P = 5%
$t_{5\%}$ *Student*'s t value belonging to i number of measuements at the probability level of P = 5%

2. Results

By means of the rapid oxidation test, it was possible to establish the minimum ground paprika and tomato seed concentration which provides an antioxidant effect approximately identical with that of 0.01% BHT. This was shown to be 5% related to fat. By further reducing the concentration the oxi-

dation inhibitory effect decreased, too. The effective concentration of the *n*-hexane extract was also determined. Results are shown in Figures and Tables.

2.1. Results of rapid oxidation experiments in the drying oven

Figure 1 illustrates the results of experiments carried out with paprika seed and its *n*-hexane extract.

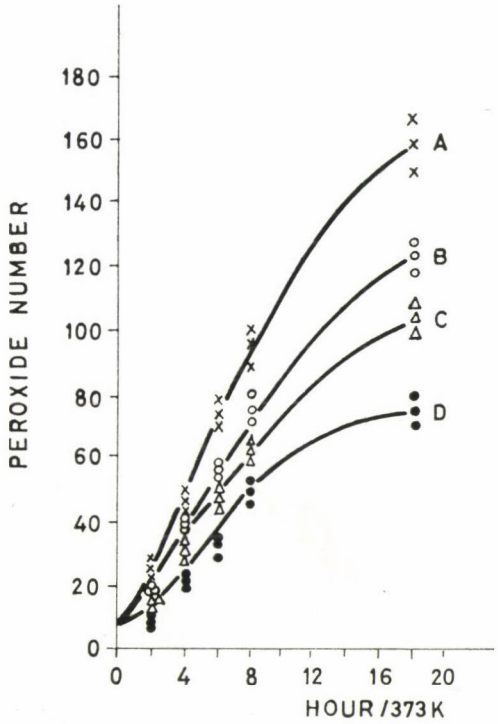

Fig. 1. Oxidation experiments of sunflower seed oil with added paprika seeds and their extracts. A: control sample of sunflower seed oil; B: sunflower seed oil + 0.01% BHT; C: sunflower seed oil + 0.5% paprika seed extract; D: sunflower seed oil + 5% ground paprika seed

As shown by the curves in sunflower seed oil, at the temperature employed, ground paprika seed ensures a higher stability than its extract.

Similar experiments were carried out with tomato seeds and their *n*-hexane extract. Results are shown in Fig. 2.

The antioxidant effect of tomato seeds and of the isolate obtained from them is similar to the effect of paprika seeds and their extract.

Figure 3 shows the effect of paprika seeds and their extract on rape seed oil.

Both ground paprika seeds and their *n*-hexane extract proved to be effective antioxidants in rape seed oil.

In Fig. 4 the antioxidant activity of ground paprika seed and its *n*-hexane extract in lard substrate is shown.

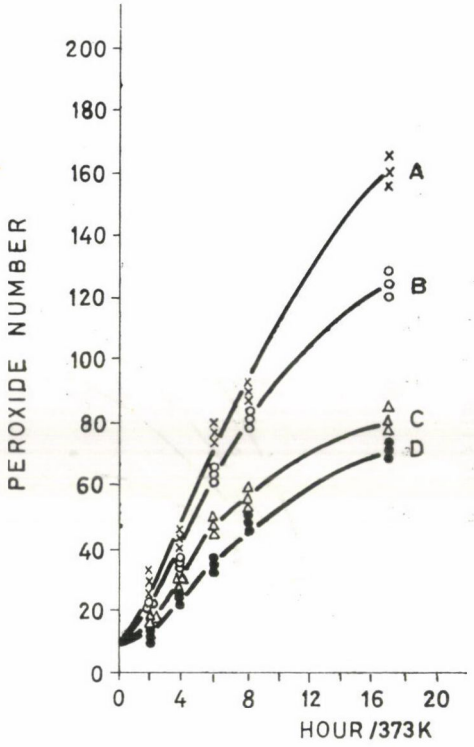

Fig. 2. Oxidation experiments of sunflower seed oil with added tomato seed and its extracts. A: control sample of sunflower seed oil; B: sunflower seed oil + 0.01%BHT; C: sunflower seed oil + 0.5% tomato seed extract; D: sunflower seed oil + 5% ground tomato seeds

Both ground paprika seeds and their extract proved to be effective in slowing down the oxidation of lard.

The average values obtained in the serial tests illustrated in the Figures are marked. The values of the coefficients of variation are below $\pm 15\%$. The evaluation by mathematical statistical methods has shown the differences between the peroxide values of the control samples and those containing ground seeds to be significant at the probability level of $P = 1\%$

2.2 Results of rapid oxidation experiments by AOM

The antioxidant activity of the seeds and their extracts in trioleate substrate was followed up by the AOM. Results expressed as the time required to reach a peroxide value of 25 (induction period) are given in Table 1.

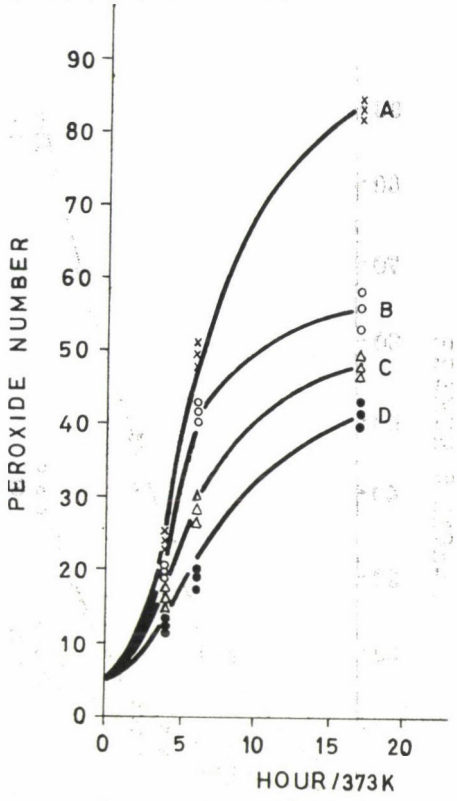

Fig. 3. Oxidation experiments of rape seed oil with added paprika seed and its extracts A: rape seed oil, control; B: rape seed oil + 0.01% BHT; C: rape seed oil + 0.5% paprika seed extract; D: rape seed oil + 5% ground paprika seed

Significant differences were established by the least significant difference ($SD_{5\%} = 1.96$) as calculated from the results of the analysis of variance. The results are given in Table 2.

As can be seen in Table 1, all additives, except the carbontetrachloride extract, had a significant effect on the inhibition of oxidation as compared with the control. In comparison to the samples containing BHT, only those containing ground seeds differ significantly. The samples with added extract are significantly worse.

In this series of experiments, too, the best results were achieved by adding ground seeds. Further efforts are needed to obtain better extracts.

2.3. Results of experiments carried out in the thermostat

In order to find the components responsible for the antioxidant effect preparation of the following pattern was carried out. True to variety tomato

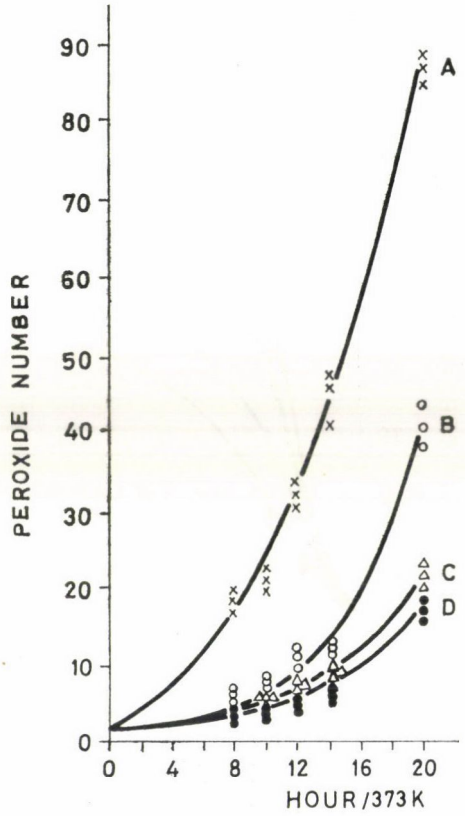

Fig. 4. Experimental oxidation of lard with paprika seeds and their extract. A: control sample of lard; B: lard + 0.01% BHT; C: lard + 0.5% paprika seed extract; D: lard + 5% ground paprika seed

seeds were ground and extracted in *n*-hexane. Tocopherols were separated by the known method (BERNDORFER-KRASZNER, 1970) and the phospholipids and neutral lipids were isolated, the total lipid content and the ground seeds after extraction were used. The oxidation experiments were carried out by adding all these products to lard. In each sample the amount of the agent corresponded to 5% ground seed. The results are shown in Fig. 5.

Table 1

Antioxidant activity as determined by the AOM method

Oxidized material	Stability AOM test (h)	Significance test	
		compared to control	compared to sample containing BHT
Trioleate without additive	2.5	–	–*
Trioleate + 0.01% BHT	12.0	+*	–
Trioleate + 5% ground paprika seed	11.5	+*	–
Trioleate + tocopherol of 5% paprika seed	5.0	+*	–*
Trioleate + 5% ground tomato seed	12 0	+*	–
Trioleate + tocopherol in 5% tomato seed	6.5	+*	–*
Trioleate + *n*-hexane extract of 5% tomato seed	8.0	+*	–*
Trioleate + *n*-hexane extract of 5% paprika seed	7.8	+*	–*
Trioleate + benzene extract of 5% tomato seed	9.0	+*	–*
Trioleate + methanol extract of 5% tomato seed	6.0	+*	–*
Trioleate + carbon tetrachloride extract of 5% tomato seed	4.0	–	–*

+* = significantly better; –* = significantly worse; – = difference non-significant

The differences between peroxide values originating from different treatments were subjected to analysis of variance. Results are shown in Tables 3, 4 and 5.

Figure 5 and the significance tests show that the best results were achieved by the use of tomato seeds. Inhibition of oxidation by tocopherols was lower and the phospholipids, separated from other components of seeds actually increased the rate of oxidation. This result was inexpected because cephalin

Table 2

Analysis of variance of the antioxidant activity as determined by the AOM method

Factors	*SQ*	*DF*	*MQ*	*F*
total	350.25	32	10.94	
treatment	321.30	10	32.13	
error	28.95	22	1.32	24.34***

*** very highly significant at the probability level of $P = 0.1\%$

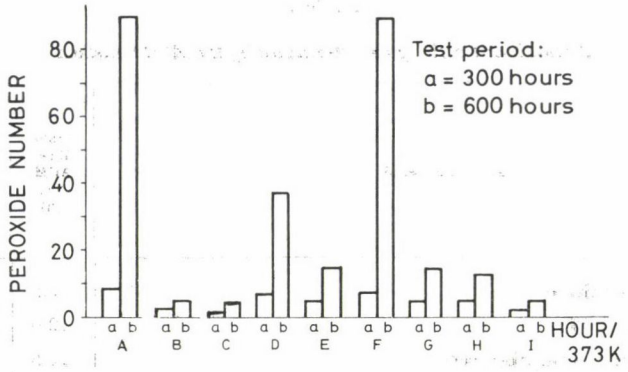

Fig. 5. Experimental oxidation of lard with added true to variety tomato seeds and their different extracts at 323 K (50 °C). A: control sample of lard; B: lard + 0.01 % BHT; C: lard + 5% ground tomato seed; D: lard + *n*-hexane extract of 5% tomato seed; E: lard + tocopherols of tomato seeds; F: lard + phospholipids of tomato seeds; G: lard + neutral lipids of tomato seeds; H. lard + total lipid of tomato seeds; I: lard + residual tomato seeds after *n*-hexane extraction

and lecithin are present among the phospholipids and these are known to be weak antioxidants, or as here, synergists. It was found interesting that the seed residue extracted with *n*-hexane was nearly as effective as the untreated ground seed or BHT. This is the more surprising as it was established by thin-

Table 3

Results of analysis of variance of data in Fig. 5 after 300 h of oxidation

Factors	*SQ*	*DF*	*MQ*	*F*
total	176.84	29	6.10	
treatment	173.04	9	19.23	
error	3.80	20	0.19	101.20***

*** very highly significant at the propability level of $P = 0.1\%$

Table 4

Results of analysis of variance of data in Fig. 5 after 600 h of oxidation

Factors	*SQ*	*DF*	*MQ*	*F*
total	37 226.20	26	1 431.77	
treatment	36 929.04	8	4 616.13	
error	297.16	18	16.51	279.59***

*** very highly significant at the probability level of $P = 0.1\%$

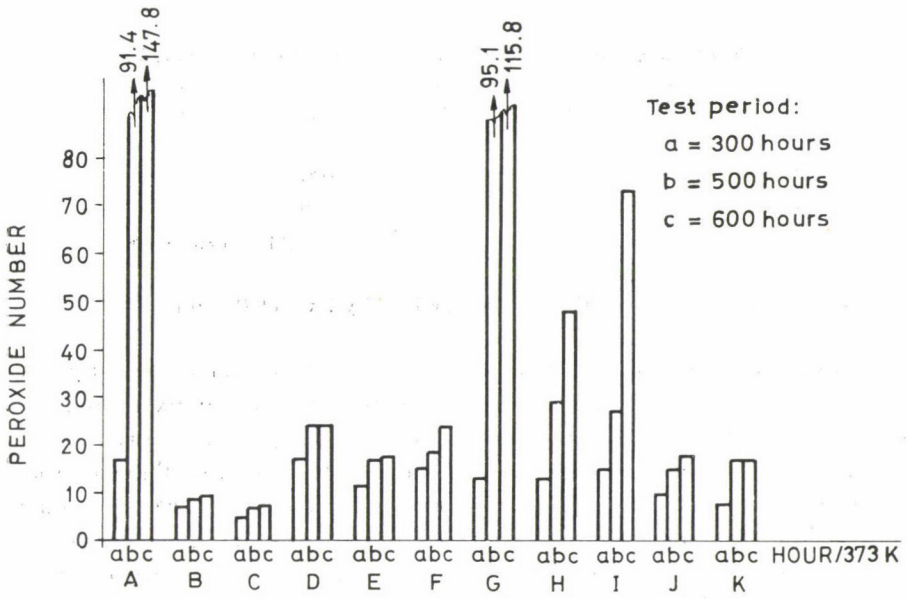

Fig. 6. Experimental oxidation of lard with added true to variety tomato seeds and their extracts with solvents of increasing polarity. A: control sample of lard; B: lard + 0.01% BHT; C: lard + 5% ground tomato seeds; D: lard + *n*-hexane extract of tomato seeds; E: lard + benzene extract of tomato seeds; F: lard + chloroform extract of tomato seeds; G: lard + methanol extract of tomato seeds; H: lard + distilled water extract of tomato seeds; I: lard + tomato seeds after extraction with different solvents; J: lard + extract of tomato seeds with solvents D-H; K: lard + tomato seeds after extraction with solvents D-H

Table 5

Results of significance tests carried out at the probability level of $P = 5\%$ between treated and control samples on one hand and treated samples and BHT stabilized samples, on the other

Treatments	Average peroxide value after 300 h	Significance test		Average peroxide value after 600 h	Significance test	
		compared to the control	compared to sample containing BHT		compared to the control	compared to sample containing BHT
A	8.53	–	–*	106.90	–	–*
B	2.30	+*	–	5.10	+*	–
C	1.93	+*	–	4.60	+*	–
D	7.17	+*	–*	37.30	+*	–*
E	4.93	+*	–*	15.40	+*	–*
F	7.70	+*	–*	92.30	+*	–*
G	5.33	+*	–*	14.20	+*	–*
H	5.40	+*	–*	14.30	+*	–*
I	2.47	+*	–	6.40	+*	–
	$SD_{5\%} = 0.74$			$SD_{5\%} = 6.93$		

+* significantly better; –* significantly worse; – difference non-significant

Table 6

Results of analysis of variance of data in Fig. 6 after 600 h oxidation

Factors	*SQ*	*DF*	*MQ*	*F*
total	29 407.55	32	918.98	
treatment	29 308.00	10	2 930.80	
error	99.35	22	4.51	649.84***

*** very highly significant at the probability level of $P = 0.1\%$

layer chromatography that the *n*-hexane extract contains the majority of tocopherols, thus, no known antioxidants remain in the extracted seeds. The residual antioxidative agents have to be of apolar character.

Results of a series of experiments are shown in Fig. 6. The same lot of ground seeds was extracted consecutively with solvents of increasing polarity. The individual extracts were evaporated under vacuum and blended with lard. Another portion of seeds was extracted with the mixture of solvents and the extract after evaporation was blended with lard. Ground seeds extracted by the various solvents were also admixed to lard. The lard samples thus prepared were then exposed to oxidation at 323 K (50 °C). The same treatment was given the control lard and the lard containing BHT.

Table 7

Results of significance tests at the probability level of $P = 5\%$

Treatments	Average peroxide values	Significance test	
		compared to control *A*	compared to control *B*
A	93.37	–	–*
B	8.57	+*	–
C	6.50	+*	–
D	24.53	+*	–*
E	16.23	+*	–*
F	18.80	+*	–*
G	93.70	+*	–*
H	27.50	+*	–*
I	27.66	+*	–*
J	16.00	+*	–*
K	17.03	+*	–*

$SD_{5\%} = 3.47$

+* significantly better; –* significantly worse; – difference non-significant

The column diagram shows that the extracts obtained with apolar solvents gave fairly good results as compared to the control after 300, 500 and 600 h of treatment. The extract obtained with methanol resulted in higher peroxide values than the extract obtained with the most polar solvent, *i.e.* water. Lard mixed with non-extracted ground seeds was also better than samples containing BHT. Samples prepared with ground seeds extracted with different solvents became oxidized at various speeds.

Peroxide values obtained after 500 h of treatment were evaluated by analysis of variance. The results are shown in Tables 6 and 7.

2.4. Autoxidation at room temperature

Storage experiments were carried out with lard obtained under industrial conditions on the ALFA-LAVAL production line. BHT, ground tomato seeds and *n*-hexane extract of tomato seeds, resp., were used as antioxidants. The lard samples homogenized with the additives and the control samples were packaged in grease-proof paper, 0.5 kg each. Results are shown in Table 8.

As can be seen in the Table, the samples with added tomato seeds were more stable than those containing BHT. Neither the BHT, nor the tomato-seed-containing samples reached, in 12 month, the rancidity threshold of 25 peroxide value units.

Table 8

Oxidation of lard at ambient temperature and light

Time	No additive		Lard + 0.01% BHT		Lard + 5% tomato seed		Lard + extract of 5% tomato seed	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
0	1.4	0.08	–	–	–	–	–	–
1	2.6	0.08	1.1	0.08	0.5	0.13	2.0	0.19
2	2.5	0.18	1.5	0.06	0.5	0.10	2.7	0.20
3	10.0	2.52	4.3	0.39	2.8	0.21	6.9	0.61
4	9.3	0.65	4.2	0.24	2.7	0.19	7.3	0.68
5	15.9	0.62	5.6	0.47	2.3	0.16	12.0	0.91
6	22.5	1.47	6.3	0.97	2.6	0.21	15.3	1.01
7	31.3	2.98	9.1	0.55	2.8	1.08	24.2	2.43
8	81.6	6.43	17.2	0.58	8.7	0.67	41.1	2.42
9	87.1	3.65	17.3	0.50	9.4	0.46	46.1	2.58
10	164.2	3.68	13.4	0.65	8.6	1.13	50.4	5.30
11	95.3	6.09	15.2	1.21	4.7	0.63	64.5	7.18
12	129.1	6.50	23.1	4.87	6.8	0.69	34.4	0.85

Notations: $\bar{x}$ **average of 3 parallel peroxide values;** s **standard deviation**

Statistical calculations have shown the peroxide values of all the treated samples to be significantly better than those of the control samples. In comparison to the samples containing BHT, those containing tomato seeds were significantly better, while those containing *n*-hexane extracts were significantly worse.

The results of the experiments show that paprika seeds and particularly tomato seeds are capable of inhibiting autoxidation in lard; 5% seeds as related to lard are equivalent to 0.01% BHT generally used.

According to other investigations, tomato seeds are more valueble both as an antioxidant and as a biological agent. Of the know antioxidants, tocopherols are present in fairly large concentration, further: ascorbic acid as an inhibitory agent, citric acid as synergist and cephalin and lecithin are also present. All these compounds may participate in the antioxidant effect. Further research into the effect of the mixture of the individually extracted agents seems desirable. It is intended to continue the experiments of extracting, identifying and establishing the antioxidant activity of these agents.

*

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BOOK REVIEWS

"Refrigeration Science and Technology" Volume 42. Cooling, freezing, storage and transport: biological and technical aspects

International Institute of Refrigeration, Paris, 1979. 454 pages.

The volume, published by the International Institute of Refrigeration, contains the proceedings of a joint meeting of three IIR Commissions, held in Budapest (Hungary) 25–28 April, 1978.

The book contains 50 reports subdivided into following subjects: Chilled storage of fruits (5 papers); Evaluating the quality of frozen and quick-frozen foods (10 papers); prepared foods (7 papers); freezing and thawing techniques (4 papers); display cases for frozen food (3 papers); cold store operations, the appreciation of storage capacity (5 papers); energy conservation in refrigerated storage (7 papers); refrigerated transport (test methods, temperature monitoring, choice of vehicles (9 papers).

Chilled storage of fruit

Apple varieties Jonathan and Golden Delicious, sliced apple for industrial processing, various apricot varieties, conventional cold storage of a banana and a pineapple variety are the topics treated in the papers.

Evaluating the quality of frozen and quick-frozen foods

The relation of quality and temperature in quick-frozen foods is discussed in four papers.

Several papers discuss the problems of quality in relation to certain quick-frozen products: spinach puree, peaches, fish, different kinds of cheese. One of the papers studies weight loss upon storage of fish as a function of the size of the store room and the refrigerating system.

Prepared foods

Economy and technical equipment of central kitchens in France are the subject of two papers. Experiences gained in Poland with the use of industrially processed prepared foods in institutional feeding are reported on. Another paper discusses a new freezing technology utilizing dry-ice. In other papers individual prepared food items, *e.g.* potato dishes, dishes containing noodles, dishes based on scallop, are described.

Freezing and thawing techniques

Subjects reported on are: freezing in dynamic dispersed medium, rotating contact freezing equipment, thawing of fish by various methods, thawing peaches by microwave treatment.

Display cases for frozen foods

The following subjects are discussed: improvement of deep-freezing cabinets, reduction of their radiation heating and the study of cooling bag.

Cold store operation: the appreciation of storage capacity

In addition to the appreciation of storage capacity, the papers report on the classification of cold stores and the determination of the time of their reconstruction.

Energy conservation in refrigerated storage

In relation to this subject the thickness of insulation, the control of suction pressure dependent on loading, the combination of cooling and heating and establishing underground cold stores are the topics of these reports. The problem of energy saving on the cold chain and refrigerator with ice storage battery are also discussed.

Refrigerated transport (test methods, temperature monitoring, choice of vehicles)

The subjects discussed are: standardization of the equipment of refrigerated transport, testing methods, view-points applied in their selection, problems related to the application of eutectic plates, problems related to temperature recording, refrigerated transport of respirating food and meat, use of containers.

E. ALMÁSI

Saving of energy in the production of cold

International Institute of Refrigeration, Paris, 1979. 267 pages.

This is the title of the new publication of the International Institute of Refrigeration (IIR) containing the proceedings of a 3-day meeting of IIR Commission B2 (Refrigerating machinery) held at Delft (The Netherlands) on 13–15 September 1978. This new publication is the 43rd in the series "Refrigeration Science and Technology" under which title the Institute publishes the proceedings of its scientific and technical Commissions. The publication contains 23 reports presented by experts of 12 countries (Belgium, Denmark, United Kingdom, USA, France, The Netherlands, Israel, Japan, German Democratic Republic, German Federal Republic, Norway, New Zealand). 20 papers are in English, three in French and each paper is accompanied by a 200-word informative summary in another language.

The reports are subdivided into 3 sections:

"Saving of energy by improvement of current refrigeration systems" (12 reports);

"Integration of refrigeration in energy saving systems" (3 papers);

"Less current systems and alternative sources of energy and cold" (6 papers).

The introductory paper was written by *P. Glandsdorff*, the chairman of the meeting, under the title: "Active and passive sides of energy requiring procedures and the analysis of losses of energy." In the paper the inevitable losses of energy in refrigeration are discussed on the basis of the basic principles of thermodynamics.

In the summary paper ("Refrigeration and saving of energy") *G. Lorentzen* gives a short review of the correlation between growth of the population and energy utilization. The review demonstrates the possibility of a natural disaster-like crisis due to the depletion of coal, petroleum and natural gas resources. The importance for the near future of the extension of the use of solar, tidal and geothermical energy is stressed. Although; the total energy requirement of refrigeration (chilling, conditioning, *etc.)* forms only about 2–3% of the total energy utilization, the saving achieved in this field is a part of the global general energy husbandry and the possibilities presented in the papers were evaluated from this aspect.

Finally a detailed prognosis of the probable proportions of utilization of various, kinds of energy till 2025 is given.

M. DEMECZKY

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PROTEIN PRODUCTION BY MICROFUNGI FROM CONVENTIONAL AND UNCONVENTIONAL CARBON SOURCES

K. ZETELAKI-HORVÁTH and K. VAS

(Received 20 January 1979, accepted 3 March 1979)

The effects of an unconventional and two conventional carbon sources (at concentration levels of 2, 4, 6, 8 and 10%) on the biomass and protein production of three microfungi *(Actinomucor, Mucor* and *Rhizopus)*, were investigated. The mycelial and protein yields of the *Actinomucor* and *Mucor* cultures were the highest in bread medium.

The carbon source input and the cost of the biomass and protein production proved to be the lowest in the *Mucor* culture.

The lowest carbon source input on unit product occurred at low (2%) carbon source concentration, the lowest production cost was found in media of higher (4, 6%) carbon source concentration.

In the fermentation industry, sucrose and glucose are often used as carbon sources in culture media. The costs of these carbon sources are rather high. Economic requirements create pressure to find substrates of low cost. The utilization of wastes of the food industry and of agriculture (CHURCH *et al.*, 1973; ADER *et al.*, 1975; IMRIE, 1975; RIGHELATO *et al.*, 1976) is important in finding cheep carbon sources which are equal to or perhaps even more valuable than the conventional ones. Since in many parts of the world there is a shortage in protein while carbohydrates can be found in abundance, the transformation of carbohydrates into protein by microorganisms is a concept of increasing importance (SPICER, 1973; SEKERI-PATRYAS *et al.*, 1973).

In our present work, an unconventional carbon source was compared with the conventional ones, like glucose and sucrose, for its suitability for biomass and protein production by three microfungi. This carbon source was a food waste, namely dried bread which occurs in large quantities (more than 5000 t per year in Budapest only). This solid raw material, used in dried, ground form, could easily be utilized by filamentous fungi.

1. Materials and methods

1.1. Microorganisms

Actinomucor repens (No. 207), *Mucor mucedo* (No. 224), *Rhizopus cohnii* (No. 222) strains (from the culture collection of the RESEARCH INSTITUTE OF VITICULTURE AND ENOLOGY, Budapest) were used in this experiment. Stock cultures were maintained on agar slants.

1.2. Nutrient media

Composition of the inoculation medium: yeast extract 25 cm^3, glucose 50 g, corn-steep liquor 10 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7\ H_2O$ 0.5 g filled up with water to 1000 cm^3; pH: 5.0.

Fermentation media consisted of the following ingredients: carbon source [glucose, sucrose or dry ground bread (water content ca. 10%)] 20, 40, 60, 80 or 100 g; corn-steep liquor 10 g; $(NH_4)_2SO_4$ 10 g; $ZnSO_4$ 0.25 g; $MnSO_4$ 0.1 g; KH_2PO_4 1.0 g filled up with water to 1000 cm^3; pH: 4.0.

1.3. Method of cultivation

Cultures were incubated in 10-l glass fermentors. The 5900-cm^3 medium in a fermentor was inoculated with 600 cm^3 of a 24-h vegetative culture. The temperature of incubation was 28 °C. The agitation speed was 460 rpm, the aeration rate: 1.0 l min^{-1} l^{-1}, ensuring an oxygen transfer rate of 49 mmol l^{-1} h^{-1} determined by the method of COOPER and co-workers (1944).

In the course of these experiments only the type and concentration of the carbon source were varied, the other parameters were fixed.

1.4. Determination of mycelial yield

Samples were taken from the cultures at 6-h intervals. Mycelia were then filtered through a nylon cloth, washed thoroughly and dried in an oven at 25 °C by ventillation. Mycelium yields were calculated from the air-dry mycelial weights (moisture content: 10%).

1.5. Protein determination

The protein content of the mycelia was determined by the modified biuret method of HERBERT and co-workers (1971).

1.6. Production costs

The production costs contain the main costs of the fermentation, *i.e.* power consumption of agitation and aeration and the cost of the carbon source.

1.7. Evaluation of the experiment

From the data of three parallel fermentations the average mycelium and protein yields were calculated. The mean values are given in the Tables.

The carbon source input on the production of unit quantity of biomass (mycelium) and protein was calculated at intervals of 6 h.

The production cost of unit quantity of biomass and protein was also calculated to comprise the cost of energy consumption of agitation and aera-

tion (ZETELAKI-HORVÁTH & VAS, 1980) and the cost of the carbon source. The mean values of three fermentations are given in the Tables.

With 460 rpm and 1.0 l min^{-1} l^{-1} aeration, 0.436 kW power was measured which resulted in 3.57, 4.76 and 5.95 kWh of energy consumption in the 36-, 48- and 60-h cultures, resp. Calculation of the cost of the energy was based on the industrial price, *i.e.* 1 Forint (Ft) per kWh.

The costs of 10 l of the medium, with carbon source concentrations of 10, 8, 6, 4 and 2%, resp., were as follows: sucrose: 9.60, 7.68, 5.76, 3.84 and 1.92 Ft; glucose: 9.97, 7.98, 5.98, 3.99 and 1.99 Ft; bread: 1.50, 1.2, 0.90, 0.60 and 0.30 Ft, resp.

2. Results

2.1. Biomass and protein production by Actinomucor repens *on different carbon sources*

2.1.1. The effect of type and concentration of carbon source on biomass yield. Mycelium yields of *Actinomucor repens* in different concentrations of glucose, sucrose and dry ground bread media as well as the carbon source consumption for the production of unit (kg) mycelium yield are summarized in Table 1, at the ages important from the point of view of harvest.

Mycelial yield of the *Actinomucor repens* culture was lowest in glucose medium and highest in bread medium. In the case of glucose, the increase in carbon source concentration from 2 to 10% resulted in a slight increase in the mycelial yield, while in the case of sucrose and ground bread, the maximal yields were measured at carbon source concentrations of 6 and 8%, resp. Further increase in the concentration of both carbon sources resulted in no increase in the biomass yield.

One kg of mycelium was produced from the highest quantity of carbon source in the case of glucose and from the lowest quantity in the case of bread, resp.

The highest mycelium yields obtained in glucose, sucrose and in bread media were 7.5, 21.5 and 37.9 g l^{-1} at carbon source concentrations of 100, 60 and 80 g l^{-1} in the 60-, 72- and the 60-h cultures, resp.

The lowest carbon source inputs for the production of 1 kg mycelium were measured at the lowest carbon source concentration (20 g l^{-1}), while the lowest production costs were established at various carbon source concentrations (2% glucose, 4% sucrose and 8% bread medium, resp.).

2.1.2. The effect of type and concentration of carbon source on protein yield. Like mycelial yields, protein yields of *Actinomucor repens* were also lowest in the case of glucose and highest in the case of bread. The increase in carbon source concentration resulted in an increase in the protein yield in the bread medium (Table 2).

Table 1

The effect of various carbon sources and their concentration on the mycelium yield, carbon source conversion (amount of carbon source per unit biomass) and unit cost of biomass of Actinomucor repens
(Cultivation conditions: see Sections 1.2–1.3)

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36		5.2	3.85	[140]	7.2	2.77	100	17.5	1.14	33
48	20	5.4	3.70	166	11.3	1.76	80	18.0	1.11	41
60		5.7	3.51	189	12.2	1.63	88	20.2	[0.99]*	45
72		5.8	[3.45]	221	13.6	[1.47]	93	17.0	1.18	65
36		5.0	8.00	187	7.0	5.71	131	16.3	2.45	37
48	40	5.4	7.41	202	15.7	2.55	70	23.0	1.74	34
60		5.7	7.02	225	19.0	2.10	[67]	22.5	1.78	42
72		6.2	6.45	235	18.6	2.15	79	19.0	2.10	59
36		5.5	10.91	206	8.0	7.50	139	18.0	3.33	35
48	60	6.0	10.00	219	17.0	3.53	76	27.5	2.18	29
60		6.5	9.23	229	19.4	3.09	75	34.0	1.76	29
72		4.5	13.33	257	[21.5]	2.79	77	31.0	1.93	37
36		5.4	14.81	242	7.0	11.43	186	19.0	4.21	34
48	80	6.0	13.30	252	15.2	5.26	97	31.1	2.57	27
60		7.0	11.43	241	21.2	3.77	78	[37.9]*	2.11	[27]*
72		6.5	12.31	274	21.1	3.79	87	35.0	2.28	34
36		5.5	18.18	279	7.8	12.82	191	16.2	6.17	42
48	100	7.0	14.28	244	16.0	6.25	105	23.0	4.35	38
60		[7.5]	13.33	252	21.7	4.61	85	32.2	3.10	32
72		6.5	15.38	318	20.3	4.93	100	34.0	2.94	36

[ ]* best among carbon sources
[ ] best among carbon source concentration levels

Table 2

The effect of various carbon sources and their concentration on the protein yield, carbon source conversion (amount of carbon source per unit protein) and unit cost of protein of Actinomucor repens
(Cultivation conditions: see Sections 1.2–1.3)

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36		2.57	7.8	[294]	2.82	7.09	264	7.58	[2.64]*	75
48		2.65	7.55	332	4.63	4.32	201	7.39	2.72	103
60	20	2.47	8.10	436	4.67	[4.28]	228	6.46	3.09	142
72		3.07	6.51	423	4.49	4.45	278	5.25	3.81	210
36		2.81	14.23	340	3.37	11.87	283	8.79	5.89	88
48		2.80	14.28	405	6.50	6.15	169	8.41	4.76	91
60	40	2.66	15.04	470	8.27	4.84	[154]	7.71	5.19	123
72		2.69	14.87	534	5.51	7.26	265	6.06	7.90	226
36		2.75	21.82	412	3.53	17.00	316	5.23	11.47	119
48		2.61	22.99	525	7.54	7.96	172	8.44	7.11	94
60	60	2.75	21.82	542	[8.58]	6.99	172	10.36	5.79	96
72		2.27	26.43	667	8.09	7.42	206	5.95	10.08	193
36		2.56	31.25	533	3.12	25.64	434	5.21	15.35	125
48		2.64	30.30	605	6.42	12.46	228	7.44	10.75	111
60	80	[3.20]	25.00	520	7.91	10.11	207	10.27	7.78	140
72		2.90	27.58	647	5.75	13.91	320	9.19	8.70	129
36		2.24	44.64	681	3.61	27.70	427	8.50	11.78	80
48		2.66	37.59	622	6.69	14.95	248	10.22	9.78	84
60	100	2.74	36.50	687	7.33	13.64	243	[15.28]*	6.54	[68]*
72		2.66	35.79	752	6.71	14.90	300	14.11	7.09	87

[ ]* best among carbon sources
[ ] best among carbon source concentration levels

A positive correlation between the concentration of carbon source and the carbon source input on unit protein yield was found in all three media used.

In glucose, sucrose and bread media, the highest protein yields of the *Actinomucor* cultures were 3.20, 8.58 and 15.28 g l^{-1}, when the concentration of the carbon source was 80, 60 and 100 g l^{-1}, resp. and the age of the cultures 60 h.

One kg of protein was produced from the lowest quantity (2.64 kg) of carbon source in bread medium, followed by the sucrose and glucose media (4.28 and 6.51 kg), resp.

The price of protein was the lowest with the 10% bread medium, while in the case of sucrose and glucose at concentrations of 4 and 2%, resp.

2.2. Biomass and protein production by Mucor mucedo *on various carbon sources*

2.2.1. The effect of type and concentration of carbon source on biomass yield. Of the three carbon sources tested, the mycelial yield of *Mucor mucedo* was the highest in the bread medium. An increase in the biomass yield was observed with the increase in bread concentration. In the case of glucose and sucrose, the highest yields occurred in media of 6 and 8% (Table 3), resp.

The quantity of carbon source consumed for the production of 1 kg biomass increased with the increase of carbon source concentration, for every carbon source tested.

The highest mycelium yields measured in *Mucor* cultures grown in glucose, sucrose and ground bread media were 24, 29 and 38.6 g l^{-1}, at concentrations of 60, 80 and 100 g l^{-1}, resp., in the 72-h cultures.

The lowest carbon source input and the highest yield had different parameters. The lowest quantity of carbon source consumed for the production of one kg of *Mucor* biomass (0.9 kg) was found in the 48-h culture, at a bread concentration of 20 g l^{-1}. The lowest glucose consumption for the production of unit biomass was 1 kg, also at the lowest carbon source concentration, but under a longer (60 h) period of incubation. The lowest carbon source input (1.78 kg) for the production of 1 kg mycelium was observed in sucrose medium at 4% sucrose concentration in the 60-h culture.

The *Mucor* biomass was cheapest in 6% bread medium. Of the glucose and sucrose concentrations used, 2 and 4% proved to be best from the point of view of production cost.

2.2.2. The effect of type and concentration of carbon source on protein yield. With the exception of sucrose, the increase in concentration of carbon source resulted in an increase in the protein yield of *Mucor mucedo*. The highest protein yields in glucose, sucrose and bread media were 6.31, 11.20 and 16.75 g l^{-1} in the 72-, 60- and 72-h cultures, resp., when the concentration of the carbon source was 100, 60 and 100 g l^{-1}, resp. (Table 4).

Table 3

The effect of various carbon sources and their concentration on the mycelium yield, carbon source conversion (amount of carbon source per unit biomass) and unit cost of biomass of Mucor mucedo
(Cultivation conditions: see Sections 1.2–1.3)

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36	20	10.0	2.00	73	8.8	2.27	83	20.2	0.99	28
48		16.0	1.25	57	9.5	2.10	84	22.2	0.90*	33
60		20.0	1.00	54	6.2	3.22	180	17.2	1.16	55
72		18.0	1.11	70	3.3	6.06	388	13.2	1.51	85
36	40	15.4	2.60	61	10.0	4.00	92	22.2	1.80	27
48		19.0	2.10	58	16.2	2.47	67	26.6	1.50	29
60		21.0	1.90	61	22.5	1.78	57	22.8	1.75	42
72		22.0	1.82	67	19.6	2.04	74	19.9	2.01	56
36	60	16.0	3.75	71	11.7	5.13	94	24.0	2.50	26*
48		21.0	2.85	62	15.4	4.48	83	28.6	2.09	28
60		23.0	2.61	65	22.5	2.67	65	31.2	1.92	31
72		24.0	2.50	69	23.3	2.57	69	29.7	2.02	40
36	80	15.0	5.33	89	13.0	6.15	100	25.3	3.16	26
48		22.0	3.64	69	15.2	5.26	97	30.8	2.60	27
60		23.0	3.48	73	25.5	3.14	65	34.5	2.32	29
72		20.0	4.00	93	29.0	2.76	63	35.5	2.25	33
36	100	16.0	6.25	96	13.0	7.70	115	23.5	4.25	29
48		19.0	5.26	90	14.8	6.76	113	30.3	3.30	28
60		20.0	5.00	94	21.8	4.59	85	36.2	2.76	29
72		20.5	4.88	101	27.3	3.66	74	36.8*	2.59	23

* best among carbon sources
best among carbon source concentration levels

Table 4

The effect of various carbon sources and their concentration on the protein yield, carbon source conversion (amount of carbon source per unit protein) and unit cost of protein of Mucor mucedo
(Cultivation conditions: see Sections 1.2–1.3)

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36	20	4.06	4.93	183	3.23	6.19	284	9.81	2.10*	59
48		6.27	3.19	146	3.27	6.12	279	9.25	2.16	80
60		3.76	5.32	291	1.06	18.87	1084	5.77	3.47	160
72		3.10	6.45	423	1.09	18.35	1263	3.74	5.35	293
36	40	4.59	8.71	208	4.30	9.30	216	10.40	3.85	57
48		4.55	8.79	247	6.22	6.43	176	11.55	3.46	67
60		4.76	8.40	272	8.45	4.73	150	8.66	4.62	109
72		6.16	6.49	235	9.36	4.27	157	7.65	5.30	151
36	60	4.47	13.42	252	4.90	12.24	221	11.94	5.02	52*
48		4.53	13.24	291	5.24	11.45	246	13.81	4.34	57
60		4.86	12.34	313	11.20	5.36	130	13.13	4.57	75
72		5.71	10.51	290	7.15	8.39	227	11.23	5.34	101
36	80	4.43	18.06	296	4.40	18.18	289	12.64	6.33	52
48		5.29	15.12	288	5.05	15.84	296	13.27	6.03	63
60		4.34	18.43	398	8.66	9.24	189	14.14	5.66	72
72		5.20	15.38	340	7.84	11.05	253	14.96	5.35	79
36	100	6.04	16.56	255	3.98	25.12	373	12.15	8.23	56
48		5.40	18.52	311	4.56	21.93	372	14.25	7.02	61
60		5.67	17.64	329	7.26	13.77	255	14.96	6.66	69
72		6.31	15.85	331	7.72	12.95	262	16.75*	5.97	73

□* best among carbon sources
□ best among carbon source concentrations

Table 5

The effect of various carbon sources and their concentration on the mycelium yield, carbon source conversion (amount of carbon source per unit biomass) and unit cost of biomass of Rhizopus cohnii
(Cultivation conditions: see Sections 1.2–1.3)

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Biomass yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36	20	10.0	2.00	73	7.0	2.85	104	14.0	1.43*	40
48		11.5	1.74	79	7.6	2.63	121	13.2	1.51	56
60		12.5	1.60	87	8.8	2.27	124	12.0	1.69	77
72		9.0	2.22	141	8.0	2.50	158	10.0	2.00	113
36	40	12.0	3.33	78	11.8	3.39	78	20.8	1.92	28*
48		18.0	2.22	62	14.8	2.70	73	19.3	2.07	40
60		19.0	2.10	67	17.0	2.35	75	13.2	3.03	72
72		16.0	2.50	92	16.2	2.47	89	12.6	3.17	90
36	60	12.5	4.80	91	9.5	6.31	117	19.8	3.03	32
48		20.0	3.00	66	10.4	5.77	123	18.2	3.30	44
60		23.0	2.60	65	17.2	3.49	85	18.5	3.24	53
72		23.5	2.55	71	18.8	3.19	88	18.0	3.33	64
36	80	14.5	5.52	92	13.0	6.15	100	23.0	3.48	28
48		20.0	4.00	76	14.0	5.71	106	25.0	3.20	33
60		23.0	3.48	73	21.0	3.81	79	21.4	3.74	47
72		25.0	3.20	75	21.2	3.77	86	21.3	3.75	56
36	100	12.0	8.33	128	12.8	7.81	117	20.0	5.00	34
48		17.0	5.88	104	14.8	6.76	113	25.5*	3.92	34
60		19.0	5.26	99	21.5	4.65	86	20.0	5.00	52
72		21.0	4.76	98	22.0	4.54	92	17.9	5.59	68

* best among carbon sources
best among carbon source concentrations

The increase in concentration of the carbon source resulted in an increasing carbon source consumption for the production of unit quantity of *Mucor* protein. The lowest carbon source input (3.2, 4.3 and 2.1 kg) for protein production by *Mucor mucedo* in glucose, sucrose and bread media was measured at carbon source concentrations of 2, 4 and 2%, resp., in the 48-, 72- and 36-h cultures, resp.

Mucor protein was produced at the lowest price (52 Ft kg^{-1}) in 6% bread medium, while in sucrose (6%) and glucose (2%) media the lowest costs were more than double of that found in bread medium.

2.3. Biomass and protein production by Rhizopus cohnii *on different carbon sources*

2.3.1. The effect of type and concentration of carbon source on mycelium yield. In *Rhizopus* cultures the mycelial yield increased with the increase of the concentration of sucrose and bread from 20 to 100 g l^{-1}, while in glucose medium glucose concentration above 80 g l^{-1} resulted in no increase of mycelium yield (Table 5).

The highest mycelial yields (25, 22 and 25.5 g l^{-1}) of *Rhizopus cohnii* were measured in 8% glucose, 10% ground bread media in the 72- and 48-h cultures, resp.

The higher the concentration of carbon source in the medium, the higher the carbon source consumption for the production of unit quantity of biomass. Lowest carbon source inputs for the synthesis, by *Rhizopus cohnii* of 1 kg biomass were 1.6 kg glucose, 2.27 kg sucrose and 1.43 kg of bread in the 60- and 36-h cultures, resp., at the lowest (20 g l^{-1}) carbon source concentration.

The lowest biomass costs in *Rhizopus* cultures were measured at 4% carbon source concentration in every carbon source tested. Among the three carbon sources optimal production cost was obtained in bread medium.

2.3.2. The effect of type and concentration of carbon source on protein yield. The protein yields of *Rhizopus cohnii* were highest (8.57, 7.07 and 11.5 g l^{-1}) in 8% glucose, 6% sucrose and in 8% bread medium, in the 72-, 60- and 48-h cultures, resp. (Table 6).

1 kg protein was produced from the lowest (2.8 kg) quantity of bread in a 2% bread medium when the age of the culture was 36 h. When glucose and sucrose were used in the medium, the lowest carbon source input for protein production was higher (4.46 and 6.49 kg, resp.) in 2% glucose and sucrose media, in the 60- and 48-h cultures.

Optimal production cost was attained in 4% bread medium during 36 h of cultivation. In sucrose and glucose media, the lowest protein costs were obtained at 6 and 4%, resp., which were more than three times higher than the cost in the bread medium.

Table 6

The effect of various carbon sources and their concentration on the protein yield, carbon source conversion (amount of carbon source per unit protein) and unit caost of protein of Rhizopus cohnii

Age of culture (h)	Concentration of carbon source (g l^{-1})	Glucose			Sucrose			Ground bread		
		Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})	Protein yield (g l^{-1})	Conversion (kg kg^{-1})	Unit cost (Ft kg^{-1})
36	20	3.38	5.92	226	2.67	7.49	264	7.05	2.83*	85
48		4.20	4.76	215	3.08	6.49	302	6.94	3.37	110
60		4.48	4.46	242	2.71	7.38	394	6.00	3.30	159
72		2.69	7.43	462	2.53	7.90	508	5.60	3.57	206
36	40	4.57	8.75	208	3.95	10.13	229	10.28	3.90	58*
48		5.99	6.66	185	3.70	10.81	293	9.31	4.30	84
60		7.40	5.40	172	4.38	9.13	300	6.65	6.01	141
72		5.86	6.82	256	3.39	11.80	6448	5.75	7.82	197
36	60	4.81	12.47	239	4.75	12.63	233	9.39	6.39	68
48		5.55	10.81	238	4.81	12.47	271	8.21	7.31	97
60		5.74	10.45	259	7.07	8.49	210	8.92	6.73	109
72		4.30	13.95	393	4.77	12.58	347	7.77	7.72	150
36	80	4.94	16.19	267	3.62	22.09	372	10.61	7.54	62
48		7.52	10.64	202	3.87	20.67	395	11.50*	6.96	72
60		7.66	10.44	218	5.96	13.42	277	8.90	8.99	112
72		8.57	9.33	209	3.60	22.22	459	7.98	10.02	149
36	100	4.35	22.99	360	3.88	25.77	398	10.88	9.19	64
48		6.66	15.01	253	4.43	22.57	372	10.56	9.47	82
60		5.74	17.42	329	5.72	17.48	322	9.82	10.18	107
72		5.90	16.95	345	4.49	22.27	451	8.78	11.39	139

* best among carbon sources

best among carbon source concentrations

3. Conclusions

The search for cheap by-products, suitable, as carbon source, for fermentation, has become a research topic of increasing importance (CHURCH *et al.*, 1972; BLOCH *et al.*, 1973).

In the present work, dry ground bread was tried as the carbon source for the production of biomass and protein with three filamentous fungi. The effect of bread was compared with two conventional carbon sources at five levels of concentration. Growth and protein production of the strains used in this work had been previously analysed kinetically in 8% glucose and sucrose and in 4% bread medium, but the bread was in a hydrolysed form, to ensure that mycelial yield be correctly determined in the early stages of the fermentation (ZETELAKI-HORVÁTH *et al.*, 1975; 1976; ZETELAKI-HORVÁTH & VAS, 1976).

In the course of the present work, ground bread proved to be a very valuable carbon source for filamentous microorganisms, capable of utilizing complex nutrients even in unhydrolysed solid form.

The parameters of the highest yields were found at high carbon source concentrations (8–10%), while lowest carbon source consumption for unit biomass was found at lower concentrations. The cost of biomass production, comprising energy consumption of agitation and aeration and that of the carbon source, were lowest at higher carbon source concentrations.

Comparison of the three carbon sources showed that biomass production of the *Mucor* and *Actinomucor* culture proved to be very highly significantly higher on bread than on sucrose or glucose (Table 7). In the *Rhizopus* culture, the use of the above three carbon sources resulted in no significant differences in the biomass yield. Protein production of the three strains was significantly higher on bread medium than in glucose and sucrose media (Table 8).

Comparison of the three organisms showed that on sucrose medium, the mycelial yield of the *Mucor* strain was very highly significantly higher than that of the other two strains. On glucose, *Mucor* was better than *Actinomucor* but not better than *Rhizopus*.

On bread, *Mucor* yields were significantly higher than *Rhizopus* yields, while there was no significant difference in the biomass yields of the *Mucor* and *Actinomucor* strains (Table 7).

The protein yield on glucose was very highly significantly higher with *Mucor* than with *Actinomucor*, but significantly lower than that with *Rhizopus*. On sucrose, *Mucor* produced a protein yield significantly higher than *Rhizopus* but it was not significantly better than *Actinomucor*. On bread, *Mucor* was a significantly better protein producer than *Actinomucor* and very highly significantly better than *Rhizopus* (Table 8).

The lowest (0.90 kg) carbon source input for unit quantity of mycelium was measured in 2% bread medium in a 48-h *Mucor* culture. That was the

Table 7

The most favourable conditions found for biomass production of Actinomucor repens. Mucor mucedo *and* Rhizopus cohnii *strains*

Strain	Carbon source (%)	Age of culture	Yield (g l^{-1})		Comparison of carbon sources		Comparison of strains	
			mean	s	t	Level of significance	t	Level of significance
Actinomucor	glucose (10)	60	7.5	1.5	28.72	***	5.53	***
	sucrose (6)	72	21.5	1.8	13.59	***	5.84	***
	bread (8)	60	37.9	1.1		0	0.61	–
Mucor	glucose (6)	72	24.0	1.0	11.74	***		0
	sucrose (8)	72	29.0	1.3	7.21	***		0
	bread (10)	72	38.6	1.9		0		0
Rhizopus	glucose (8)	72	25.0	5.2	0.14	–	0.32	–
	sucrose (10)	72	22.0	0.6	0.95	–	8.47	***
	bread (10)	48	25.5	3.5		0	5.73	***

0 basis for comparison * significant ($P \geq 95\%$) s = standard deviation
– not significant ($P \leq 95\%$) ** highly significant ($P \geq 99\%$)
*** very highly significant ($P \geq 99.9\%$)

Table 8

The most favourable conditions found for protein yields of Actinomucor repens, Mucor mucedo *and* Rhizopus cohnii *strains*

Strain	Carbon source (%)	Age of culture	Yield (g l^{-1})		Comparison of carbon sources		Comparison of strains	
			mean	s	t	Level of significance	t	Level of significance
Actinomucor	glucose (8)	60	3.20	0.43	24.22	***	5.41	***
	sucrose (6)	60	8.58	1.58	6.63	***	1.48	–
	bread (10)	60	15.28	0.75		0	2.26	*
Mucor	glucose (10)	72	6.31	0.90	14.68	***		0
	sucrose (6)	60	11.20	2.63	3.48	*		0
	bread (10)	72	16.75	0.84		0		0
Rhizopus	glucose (8)	72	8.57	1.13	3.43	*	2.71	*
	sucrose (6)	60	7.07	1.25	4.85	***	2.45	*
	bread (8)	48	11.50	0.96		0	7.12	***

0 basis for comparison * significant ($P \geq 95\%$) s = standard deviation
– not significant ($P < 95\%$) ** highly significant ($P \geq 99\%$)
*** very highly significant ($P \geq 99.9\%$)

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Table 9

Comparison of conversion and costs for biomass production of Actinomucor, Mucor *and* Rhizopus *strains on different carbon sources*

Fungus	Carbon source conversion			Production costs				Comparison of costs on different carbon sources (using the same strain)		Comparison of costs with strains (on the same carbon source)	
	(kg kg^{-1})	Carbon source (%)	Age of culture (h)	Carbon source (%)	Age of culture (h)	Average cost	s	t	Level of significance	t	Level of significance
						Ft kg^{-1}					
	3.45	glucose (2)	72	2	36	140	33.60	5.86	***	4.34	**
Actinomucor repens	1.47	sucrose (2)	72	4	60	67	6.00	11.68	***	2.44	*
	0.99	ground bread (2)	60	8	60	27	0.60		0	0.41	–
	1.00	glucose (2)	60	2	60	54	7.00	6.80	***		0
Mucor mucedo	1.78	sucrose (4)	60	4	60	55	7.00	6.82	***		0
	0.90*	ground bread (2)	48	6	36	26*	1.20		0		0
	1.60	glucose (2)	60	4	48	62	8.90	5.85	***	1.22	–
Rhizopus cohnii	2.27	sucrose (2)	60	4	48	73	3.97	11.29	***	2.90	*
	1.43	ground bread (2)	36	4	36	28	4.51		0	0.86	–

□* best among strains

□ best among carbon sources – for one strain

0 = basis for comparison
– = not significant ($P < 95\%$)
* = significant ($P \geq 95\%$)
** = highly significant ($P \geq 99\%$)
*** = very highly significant ($P \geq 99.9\%$)

s = standard deviation

Table 10

Comparison of conversion and costs for protein production of Actinomucor, Mucor *and* Rhizopus *strains on different carbon sources*

Fungus	Carbon source conversion			Production costs				Comparison of costs on different carbon sources (using the same strain)		Comparison of costs with strains (on the same carbon source)	
	(kg kg^{-1})	Carbon source (%)	Age of culture (h)	Carbon source (%)	Age of culture (h)	Average cost	s	t	Level of significance	t	Level of significance
						Ft kg^{-1}					
	6.51	glucose (2) —	72	2	36	294	70.4	5.02	***	3.37	*
Actinomucor repens	4.06	sucrose (2)	54	4	60	154	50.0	2.97	*	1.43	–
	2.64	ground bread (2)	36	10	60	68	3.0		0	5.70	***
	3.19	glucose (2)	48	2	48	146	11.0	11.87	***		0
Mucor mucedo	4.27	sucrose (4)	72	4	60	130	18.0	4.94	***		0
	2.04*	ground bread (2)	48	6	36	52*	1.6		0		0
	4.46	glucose (2)	60	4	60	172	38.5	6.41	***	2.48	*
Rhizopus cohnii	6.49	sucrose (2)	36	6	60	211	37.5	5.42	***	4.23	**
	2.83	ground bread (2)	36	4	36	59	17.5		0	0.03	–

□* best among strains

□ best among carbon sources – for one strain

0 = basis for comparison
– = not significant ($P \leq 95\%$)
* = significant ($P \geq 95\%$)
** = highly significant ($P \geq 99\%$)
*** = very highly significant ($P \geq 99.9\%$)

s = standard deviation

Table 11

Comparison of the protein yields of the investigated strains on various carbon sources in cases when the protein yields showed highest values and the production costs gave the lowest values

Strain	Highest yields			Lowest costs			Level of significance of difference between protein production under the two conditions
	Carbon source (%)	Protein ($g\ l^{-1}$)	Age of culture (h)	Carbon source (%)	Protein ($g\ l^{-1}$)	Age of culture (h)	
Mucor	glucose (10)	6.31	72	glucose (2)	6.27	48	–
	sucrose (6)	11.20	60	sucrose (4)	11.20	60	–
	bread (10)	16.75	72	bread (6)	11.94	36	***
Rhizopus	glucose (8)	8.57	72	glucose (4)	4.48	60	**
	sucrose (6)	7.07	60	sucrose (6)	7.07	60	–
	bread (8)	11.50	48	bread (4)	10.28	36	–
Actinomucor	glucose (8)	3.20	60	glucose (2)	2.57	36	–
	bread (10)	15.28	60	bread (10)	15.28	60	–

– not significant
** highly significant
*** very highly significant

best value with the investigated strains from the point of view of carbon source consumption on unit biomass (Table 9).

When the lowest values of the production cost were compared, the bread medium proved to be the best at a very highly significant level.

The fungal biomass could be produced at the lowest price on bread medium in all the three cultures tested and no significant differences were found between the production costs with the three organisms.

The production cost of *Actinomucor* biomass was highly significantly and significantly higher on glucose and sucrose media than in the *Mucor* culture. The production costs of the *Mucor* and *Rhizopus* biomass differed significantly when sucrose was used as the carbon source.

The best carbon source for the lowest carbon source input on unit protein production of all three investigated strains proved to be again the bread medium. Protein was produced from the lowest amount of carbon source (2.04 kg) in the 42-h *Mucor* culture at 2% bread concentration (Table 10).

Bread proved to be the carbon source of choice for protein production at the lowest cost (52 Ft kg^{-1}). Among the strains tested, *Mucor mucedo* produced protein at a very highly significantly lower cost than the *Actinomucor* culture, with a cultivation period 24 h shorter, and a bread concentration 4% lower, than those of the *Actinomucor* culture.

No significant difference was found between the costs of protein production of the *Mucor* and *Rhizopus* culture. The shortest time of cultivation necessary for the cheapest production proved also identical, only the carbon source concentration was found less by 2% in *Rhizopus* culture than with the *Mucor* culture.

When the maximal protein yields were compared with the protein yields belonging to the minimal production costs, significant differences were found in only two cases (Table 11). The protein yields belonging to the lowest cost were very highly significantly lower in the *Mucor* culture on bread and significantly lower in the *Rhizopus* culture on glucose than the maxima of their protein yields.

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ENERGY REQUIREMENTS FOR AGITATION AND AERATION IN PROTEIN PRODUCTION BY MICROFUNGI ON THE LABORATORY SCALE

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The effect of three speeds of agitation and three rates of aeration were tested, at nine levels of oxygen transfer rate, on the mycelial and protein yields of three microfungi *(Actinomucor, Mucor* and *Rhizopus)* and the economics of their production at three periods (36, 48 and 60 h) of cultivation, important from the point of view of yield.

The highest mycelium and protein yields (38.5 and 14.9 g l^{-1}) were obtained in the *Mucor* culture (but at the very high oxygen transfer rate of 179 mmol l^{-1} h^{-1}). From the point of view of economics of mycelium production the use of a *Mucor* strain proved again the best (57.2 g mycelium/kWh). Studying economics of protein production showed that the protein production of *Rhizopus* strain was the most favourable (23.6 g protein/kWh) for its maximal production was obtained at a low oxygen transfer rate (49 mmol l^{-1} h^{-1}).

Aeration and agitation have been subjects of scientific investigation for several decades already. The role of oxygen, its solubility, absorption rate and the factors influencing the absorption of O_2 were described in many publications (CHAIN *et al.*, 1952; FINN, 1954; SOLOMONS & WESTON, 1961; AIBA *et al.*, 1965; BROWN, 1970). The effects of aeration and agitation on product formation were studied in different fields of the fermentation industry (DEINDOERFER & GADEN, 1955; STEEL & MAXON, 1962; HIROSE *et al.*, 1968; ZETELAKI-HORVÁTH *et al.*, 1978; DE SANTIS *et al.*, 1976).

In the last few years, the economics of agitation and aeration came to the fore (HASHIMOTO *et al.*, 1973; TAKEI *et al.*, 1975; FUJITA & HASHIMOTO, 1975), as all over the world energy economy became a question of great importance.

In the present work, the energy requirements of agitation and aeration were examined, at a rather wide range of oxygen transfer rates, on the biomass and protein synthesis of three different fungi.

1. Materials and methods

1.1. Microorganisms

A *Mucor mucedo* (No. 224) a *Rhizopus cohnii* (No. 222) and an *Actinomucor repens* (No. 207) strain were used in this experiment. The strains were kindly put at our disposal by Ms. Edit NYERGES from the culture collection of the RESEARCH INSTITUTE OF VITICULTURE AND ENOLOGY, Budapest.

1.2. Cultivation conditions

Cultivation was carried out in 10-l glass fermentors each containing 5.9 l of the medium. This volume was inoculated with 600 ml of 24-h vegetative culture. The temperature of incubation was 28 °C. Agitation (460, 700 and

Table 1

Oxygen transfer rate and power requirement of agitation and aeration in 10-l KUTÉSZ fermentors

O_2 transfer rate	Agitation			Aeration	
	rpm	power requirement for 6 fermentors (kW)	1 min^{-1} l^{-1}	air consumption for 1 fermentor (l h^{-1})	power requirement for 6 fermentors (kW)
26	460	0.436	0.5	198	0.080
49	460	0.436	1.0	396	0.159
60	460	0.436	1.5	594	0.238
85	700	0.452	0.5	198	0.080
93	940	0.470	0.5	198	0.080
100	700	0.452	1.0	396	0.159
126	940	0.470	1.0	396	0.159
135	700	0.452	1.5	594	0.238
179	940	0.470	1.5	594	0.238

940 rpm) and aeration (0.5, 1.0 and 1.5 l l^{-1} min^{-1}) conditions were studied and suitable conditions determined.

Oxygen transfer rates (OTR, determined with the method of COOPER and co-workers, 1944) under various conditions of agitation and aeration are summarized in Table 1.

Composition of the media. Inoculation medium: yeast extract 25 cm^3, glucose 50 g, corn steep liquor 10 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7\ H_2O$ 0.5 g, filled up with water to 1000 cm^3; pH: 5. *Fermentation medium:* ground bread (water content about 10%) 40 g, corn steep liquor 10 g, $(NH_4)_2SO_4$ 10 g, $ZnSO_4$ 0.25 g, $MnSO_4$ 0.1 g, filled up with water to 1000 cm^3, pH: 4.0.

1.3. Determination of mycelial yield

At certain intervals, samples were taken from the culture. The mycelia of 100 ml culture were filtered through a nylon cloth, washed from the residues of the media and dried in an oven at 105 °C to constant weight.

1.4. Determination of protein yield

Protein content of the mycelia were analysed by the modified biuret-method of HERBERT and co-workers (1971), and the protein yield was calculated from this and the data of mycelial yield.

1.5. Selection of suitable agitation and aeration conditions

Energy consumed for the production of a unit quantity of product, as well as quantity of product produced by unit energy consumption were investigated as a function of the following factors:

- speed of agitation
- rate of aeration
- period of cultivation

The other variables were fixed during the experiment.

1.5.1. Energy consumption of agitation. In the course of the experiments, agitators of six fermentors were driven by a common driving shaft. The energy consumed by agitation of the six fermentors at various speeds of agitation are also given in Table 1. Data were measured in A and converted into W according to the following equation:

$$P_{(W)} = 3 \cdot I \cdot U \cdot \cos \varphi$$

where: I = current intensity (A)
U = voltage (V)
$\cos \varphi$ = loss factor

1.5.2. Energy consumption of aeration. From the air flow (1 h^{-1}) of one fermentor and from the average pressure (bar) of air, the energy consumption of aeration was calculated as follows:

air-stream in the case of maximal consumption:

S_1 (for 1 fermentor) = 594 l h^{-1}
S_6 (for 6 fermentors) = 3564 l h^{-1} = 0.99×10^{-3} m^3 s^{-1}

air pressure:

$\Delta p = 1.75$ bar

net power:

$S\Delta p = 0.99 \times 10^{-3}$ m^3 $s^{-1} \times 17.15 \times 10^4$ N m^{-2} = 169.8 W = 0.1698 kW

Assuming 70% efficiency, a

gross power of 0.242 kW was obtained.

Data in Table 1 clearly show that the increase of aeration rate resulted in a considerable increase in power requirement.

1.5.3. Energy input on the products. The energy consumed for the production of a unit quantity of mycelium or protein and the quantity of mycelium and enzyme produced by unit energy consumption were calculated from the measured electrical values and from the time of cultivation.

2. Results

2.1. Actinomucor repens

2.1.1. Mycelium yield as a function of oxygen transfer rate. Oxygen transfer rate, ensured by various agitation and aeration conditions, was chosen to investigate the common effect of the latter factors.

The mycelial yields of 36-, 48- and 60-h *Actinomucor repens* cultures were studied as a function of the OTR. The age of the culture is of importance from the point of view of economics when determining the most suitable time of the harvest (Table 2).

The highest mycelial yield (22.3 g l^{-1}) was measured in the 36-h culture at an OTR of 60 mmol l^{-1} h^{-1} (Table 2). The second best yield was obtained in the 48-h culture at a very low OTR (26 mmol l^{-1} h^{-1}), but yields, about 20 g l^{-1}, were obtained in a rather wide range of OTR (26–93 mmol l^{-1} h^{-1}). Mycelium yields were the lowest in the 60-h cultures, indicating that autolysis may already have started. Maximum yields in the 60-h cultures were measured at an OTR of 60 mmol l^{-1} h^{-1}.

2.1.2. Actinomucor mycelium produced by unit energy. The mycelium yield produced by unit energy decreased as a function of OTR and decreased with the increase in the time of cultivation (Table 2).

The highest amounts of mycelia (38.1 and 36.5 g) produced by 1 kWh were measured in the 36-h culture. The first and second peaks were obtained at OTR of 26 and 60 mmol l^{-1} h^{-1}. Two rather high yields (34.6 and 33.2 g) were produced by unit energy in the 48-h culture, too, at OTRs of 26 and 85 mmol l^{-1} h^{-1}, resp. At both OTRs, the rate of aeration was the lowest (0.5 l l^{-1} min^{-1}) resulting in an advantageous energy input. Mycelium yields produced by unit energy were the lowest in the 60-h culture at every OTR tested.

The power input on 1 kg of mycelium was also calculated. Data are given in Table 2.

The lowest power inputs on the production of 1 kg *Actinomucor* mycelium were 26.2 and 27.4 kWh in the 36-h culture. The lowest power inputs in the 48- and 60-h cultures were 29 and 42.7 kWh at an OTR of 26 mmol l^{-1} h^{-1}.

2.1.3. Protein yield of Actinomucor as a function of oxygen transfer rate. The maxima of protein yields of *Antinomucor repens* were measured at OTRs

Table 2

Mycelium yield and energy consumption in biomass production by Actinomucor repens
(For cultivation conditions: see Section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	93	100	126	135	179	*t*	*s*
	Mycelium yield (g l^{-1})										
36	17.9	17.9	[22.3]+	13.8	15.9	14.0	14.0	13.6	13.6	2.6	0
48	[21.6]	19.4	19.5	20.9	20.7	15.9	16.0	16.0	16.0	0.3	0.4$^-$
60	18.3	17.9	[20.1]	13.5	14.1	14.5	14.1	15.0	15.1	2.9	1.0$^-$
	Mycelium yield produced by unit energy input [g(kWh)$^{-1}$]										
36	[38.1]+	33.2	36.5	29.1	31.7	26.0	24.4	22.3	21.1	2.6	0
48	[34.6]	26.9	23.9	33.2	30.9	22.1	21.8	19.6	18.7	4.6	1.4$^-$
60	[23.4]	19.9	19.7	17.1	16.9	16.1	14.8	14.7	14.1	6.4	6.4*
	Energy input on the production of 1 kg mycelium (kWh)										
36	[26.2]+	30.1	27.4	34.3	31.5	38.5	40.9	44.9	47.3	3.6	0
48	[29.0]	37.1	41.8	30.1	32.4	45.3	45.9	50.9	53.6	3.5	1.3$^-$
60	[42.7]	50.3	50.6	58.6	59.3	62.1	67.7	67.9	71.0	5.5	5.2**

[ ] highest value in the horizontal row

[ ]+ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
$^-$: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99\%$)

and at ages of cultivation different from those giving highest mycelium yields (Table 3).

The highest protein yield (11.3 g l^{-1}) was obtained in 48 h of cultivation at an OTR of 93 mmol l^{-1} h^{-1}. In the 36-h cultures, the same OTR proved to be best, too, with a protein yield of 9.9 g l^{-1}. With the increase in OTR, a slight increasing tendency was observed in the protein yields of the 60-h culture. The highest protein yield (9.7 g l^{-1}) was obtained at an OTR of 179 mmol l^{-1} h^{-1} (Table 3).

2.1.4. Actinomucor protein, produced by unit energy. According to our results, the highest protein yield that can be produced by 1 kWh, was obtained under the shortest cultivation period. When the protein produced by unit energy was examined as a function of OTR 85–93 mmol l^{-1} h^{-1} proved to be best at all the three ages of the culture tested. 19.8 and 16.9 g of protein could be produced by 1 kWh at an OTR of 93 mmol l^{-1} h^{-1} in the 36-h and 48-h cultures, resp., while in the 60-h culture, at an OTR of 85 mmol l^{-1} h^{-1} (Table 3), the protein yield was only 10.1 g.

The lowest energy input on the production of 1 kg protein was measured in the 36-h culture. Obviously the prolongation of the cultivation period resulted in an increase of energy input on the unit quantity of product. In the 36- and 48-h culture 1 kg protein could be produced by the lowest energy input (50.6 and 59.9 kWh) at an OTR of 93 mmol l^{-1} h^{-1}, while in the 60-h culture an OTR of 85 mmol l^{-1} h^{-1} gave the best result, but the energy input was much higher (98.9 kWh) than those for the younger cultures.

2.2. Mucor mucedo

2.2.1. Mycelial yields as a function of oxygen transfer rate. Mycelial yields of the 36-, 48- and 60-h cultures increased with the increase of the OTR. The only exception was the 48-h culture when the mycelial yield decreased with the increase of OTR from 146 to 179 mmol l^{-1} h^{-1} (Table 4).

In the lower range of OTR (26–100 mmol l^{-1} h^{-1}) the highest mycelial yields were measured in the 48-h cultures, while in the upper range (126–179 mmol) the 60-h cultures gave the best results. The highest mycelium yields of the 48- and 60-h cultures were 34.8 and 38.5 g l^{-1} at OTRs of 126 and 179 mmol l^{-1} h^{-1}, resp.

2.2.2. Mucor mycelium produced by unit energy. The mycelium quantity produced by 1 kWh was the highest in the 36-h *Mucor* culture, with a maximum (57.2 g) at an OTR of 85 mmol l^{-1} h^{-1}. The same OTR proved to be best in the 48- and 60-h cultures, with maxima of 54.4 and 41.1 g, resp. The lowest quantities of mycelia were produced by unit energy in the 60-h cultures (Table 4).

Table 3

Protein yield and energy consumption in protein production by Actinomucor repens
(For cultivation conditions: see Section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	91	100	126	135	179	*s*	*t*
	Protein yield ($g\ l^{-1}$)										
36	6.9	7.5	9.4	8.4	[9.9]	8.6	7.6	9.0	7.9	0.3	1.6⁻
48	7.5	9.1	8.3	10.6	[11.3]+	8.6	9.2	9.0	8.5	1.5	0
60	6.7	7.1	8.3	8.0	7.0	8.7	9.0	8.6	9.7	0.3	1.8⁻
	Protein yield produced by unit energy input [$g(kWh)^{-1}$]										
36	14.7	13.9	15.4	17.7	[19.8]+	16.5	13.3	14.7	12.3	0.3	0
48	12.0	12.6	10.2	16.8	[16.9]	11.9	12.5	11.0	9.9	0.7	10.6***
60	8.6	7.9	8.1	[10.1]	8.4	9.7	9.4	8.4	9.0	0.4	26.5***
	Energy input on the production of 1 kg mycelium (kWh)										
36	68.1	71.9	65.0	56.4	[50.6]+	62.7	75.4	67.9	81.5	6.0	0
48	83.6	79.1	98.2	58.4	[59.9]	83.7	79.8	90.5	100.8	5.0	3.4*
60	116.7	126.8	122.6	[98.9]	119.4	103.4	106.0	118.4	110.5	12.0	7.1**

[ ] highest value in the horizontal row

[ ]+ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
⁻: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99\%$)
***: very highly significant ($P \geq 99.9\%$)

Table 4

Mycelium yield, and energy consumption in biomass production by Mucor mucedo
(For cultivation conditions: see section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	93	100	126	135	179	*s*	*t*
	Mycelium yield (g l^{-1})										
36	24.7	25.1	27.1	27.1	27.5	27.6	27.8	31.5	[33.3]	1.5	5.6***
48	31.0	33.0	31.2	34.3	32.0	34.2	[34.8]	34.5	32.2	3.0	2.1*
60	22.2	24.1	30.0	32.5	31.9	33.2	36.3	34.7	[38.5]+	0.5	0
	Mycelium yield produced by unit energy input [g(kWh)$^{-1}$]										
36	52.5	46.6	44.3	[57.2]+	54.9	51.2	48.5	51.5	51.7	2.5	0
48	49.5	45.8	38.3	[54.4]	47.8	47.5	47.4	42.3	37.6	1.2	4.8**
60	28.4	26.8	29.5	[41.1]	38.2	36.9	38.0	34.1	35.9	4.3	6.4**
	Energy input on the production of 1 kg mycelium (kWh)										
36	19.0	21.5	22.5	[17.5]+	18.2	19.5	20.6	19.4	19.3	0.8	0
48	20.2	21.8	26.1	[18.3]	20.9	21.0	21.1	23.6	26.6	0.3	4.6**
60	35.2	37.3	33.9	[24.3]	26.2	27.1	26.3	29.3	27.8	1.8	6.5**

[ ] highest value in the horizontal row

[ ]+ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
⁻: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99\%$)
***: very highly significant ($P \geq 99.9\%$)

When evaluating the energy input on 1 kg mycelium as function of the OTR, 85 mmol l^{-1} h^{-1} proved to be optimal in the case of all the three cultivation periods. The lowest energy input (17.5 kWh) on the production of 1 kg mycelium was found in the 36-h culture, which was almost equal to that of the 48-h culture, while the energy input on the unit quantity of mycelium was the highest (24.3 kWh) in the 60-h culture.

2.2.3. Protein yield of Mucor as a function of oxygen transfer rate. Protein yields of *Mucor mucedo* increased with the increase of OTR from 26 to 126 mmol l^{-1} h^{-1} in the 60-h culture. In the range of 26–60 mmol l^{-1} h^{-1}, the protein yields of the 36- and 48-h culture proved to be higher than that of the 60-h culture, while in the range of 85–179 mmol l^{-1} h^{-1} the 60-h culture gave the highest yield with a maximum of 14.9 g l^{-1}. The highest values of the 36- and 48-h culture were 12.6 and 13.2 g l^{-1} at OTRs of 60 and 49 mmol l^{-1} h^{-1}, resp. (Table 5).

2.2.4. Mucor protein produced by unit energy. It is obvious that the quantities of *Mucor* protein produced by unit energy were the highest in the case of the shortest cultivation period, and decreased with the increase of the age of the culture (Table 5). The highest protein yields produced by 1 kWh (22.5 g and 14.8 g) were measured at the OTRs of 26 and 126 mmol l^{-1} h^{-1} in the 36-h and 60-h cultures, resp. In the 48-h culture, two nearly identical peaks (17.2 and 17.7 g) were found at OTRs of 26 and 126 mmol l^{-1} h^{-1}, resp.

The lowest energy input (44.3 kWh) on the production of 1 kg protein was found in the 36-h culture at OTR of 26 mmol l^{-1} h^{-1}, which was followed by the best values for the 48-h and 60-h cultures (56.5 and 67.7 kWh, resp.) at an OTR of 126 mmol l^{-1} h^{-1}.

2.3. Rhizopus cohnii

2.3.1. Mycelial yields as a function of oxygen transfer rate. Mycelium yield of the *Rhizopus cohnii* increased with the increase of the age of the culture (from 36 to 60 h) at nearly every OTR tested. There were only two exceptions in the 48-h cultures at OTRs of 26, 49 and 60 mmol l^{-1} h^{-1}, where higher yields occurred (31.1, 34.8 and 36.5 g, resp.) than those in the 60-h culture (Table 6).

The highest mycelium yields in the 36-, 48- and 60-h cultures (29.1, 36.5 and 34.5 g l^{-1}) were measured at OTRs of 60 and 49 mmol l^{-1} h^{-1}, resp.

2.3.2. Rhizopus mycelium produced by unit energy. The quantity of mycelium produced by 1 kWh showed a remarkable decrease with the increase of the OTR from 26 to 100 mmol l^{-1} h^{-1}, while at higher OTRs the decreasing tendency became lower. Oxygen transfer rates (26 and 49 mmol l^{-1} h^{-1}), proved to be best for the mycelial yields produced by unit energy in the case of all the three ages (36-, 48- and 60-h) of the cultures (56.4, 49.7 and 36.3 g, resp.).

Table 5

Protein yield, and energy consumption in protein production by Mucor mucedo
(For cultivation conditions: see Section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	93	100	126	135	179	*s*	*t*
	Protein yield (g l^{-1})										
36	10.6	10.3	11.2	8.9	10.5	11.5	12.3	**12.6**	11.0	1.1	3.5*
48	10.8	12.2	10.7	10.0	9.9	11.2	13.0	**13.2**	10.9	2.7	1.0⁻
60	7.6	8.0	8.3	10.6	11.2	11.4	14.1	12.9	**14.9**⁺	0.3	0
	Protein yield produced by unit energy input [g(kWh)$^{-1}$]										
36	**22.5**⁺	19.1	18.3	18.8	21.0	21.3	21.5	20.6	17.1	2.1	0
48	17.2	16.9	13.1	15.9	14.8	15.5	**17.7**	16.2	12.7	1.4	5.9**
60	9.7	8.9	8.1	13.4	13.4	12.7	**14.8**	12.7	13.9	2.8	2.8*
	Energy input on the production of 1 kg protein (kWh)										
36	**44.3**⁺	52.3	54.5	53.3	47.7	46.9	46.6	48.5	58.5	0.8	0
48	58.0	59.0	76.4	63.0	67.7	64.3	**56.5**	61.7	57.5	1.0	21.1***
60	102.9	112.5	122.6	74.6	74.6	78.9	**67.7**	78.9	71.9	4.0	10.3***

[ ] highest value in the horizontal row

[ ]⁺ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
⁻: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99$)
***: very highly significant ($P \geq 99.9\%$)

Table 6

Mycelium yield, and energy consumption in biomass production by Rhizopus cohnii
(For cultivation conditions: see Section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	93	100	126	135	179	*s*	*t*
	Mycelium yield (g l^{-1})										
36	26.5	28.6	[29.1]	18.9	17.6	19.0	18.0	18.8	19.3	1.1	7.7**
48	31.1	34.8	[36.5]$^{+}$	22.0	23.5	22.9	22.4	24.5	22.5	1.2	0
60	28.8	[34.5]	32.2	30.5	25.0	30.7	29.0	28.0	26.3	1.0	2.2^{-}
	Mycelium yield produced by unit energy input [g(kWh)$^{-1}$]										
36	[56.4]$^{+}$	53.1	47.6	39.9	35.1	35.2	31.4	30.8	30.0	3.6	0
48	[49.7]	48.3	44.8	34.9	35.1	31.8	30.5	30.1	26.2	4.7	2.5^{-}
60	36.8	[38.3]	31.6	38.6	29.9	34.1	30.4	27.5	24.5	1.5	20.9***
	Energy input on the production of 1 kg mycelium (kWh)										
36	[17.7]$^{+}$	18.8	21.0	25.1	28.5	28.4	31.8	32.5	33.4	4.1	0
48	[20.1]	20.7	22.3	28.6	28.5	31.4	32.8	33.3	38.1	9.2	1.4^{-}
60	27.1	[26.1]	31.6	25.9	33.4	29.3	32.9	36.4	40.8	0.2	72.7***

[ ] highest value in the horizontal row

[ ]$^{+}$ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
$^{-}$: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99\%$)
***: very highly significant ($P \geq 99.9\%$)

The lowest energy input on the production of 1 kg mycelium was measured at the lowest OTR (26 mmol $l^{-1} h^{-1}$). The energy input increased with the increase of the OTR and with the age of the culture as well. The lowest energy input measured in the 36-, 48- and 60-h cultures were as follows: 17.7, 20.1 and 26.1 kWh (Table 6).

2.3.3. Protein yield of Rhizopus as a function of oxygen transfer rate. The highest protein yield of *Rhizopus cohnii* was found in the 60-h culture (13.1 g l^{-1}) at an OTR of 49 mmol $l^{-1} h^{-1}$, but at OTRs of 85, 100 and 126 mmol $l^{-1} h^{-1}$, the protein yields were also good exceeding 11.5 g l^{-1}. In the 48- and 36-h cultures, the highest values (12.6 and 11.9 g l^{-1}) of the protein yield were measured at OTRs of 60 and 49 mmol $l^{-1} h^{-1}$, resp. (Table 7).

2.3.4. Rhizopus protein produced by unit energy. The highest protein production by 1 kWh was measured when the cultivation period was the shortest (36 h), while the lowest quantity of protein was produced by unit energy when the cultivation period was the longest (60 h) at all OTRs tested.

The quantity of protein produced by 1 kWh decreased with the increase of the OTR at all ages of the cultures tested. The only exception was the 60-h culture, where two peaks were found [14.5 and 14.7 g$(kWh)^{-1}$] at OTRs of 49 and 85 mmol $l^{-1} h^{-1}$, resp. The maxima of protein yields produced by 1 kWh were 23.6 and 16.5 g$(kWh)^{-1}$ in the 36- and 48-h cultures at OTRs of 26 and 49 mmol $l^{-1} h^{-1}$, resp. (Table 7).

Energy input necessary for the production of 1 kg protein was the lowest in the 36-h culture and increased with the increase of the OTR. The same tendency was found also in the 48-h culture. In the 60-h culture, where the highest energy was consumed for the production of 1 kg of mycelium, a fluctuation of energy input was observed as function of the OTR due to the changes in protein yield of the culture.

The lowest energy input for protein production was achieved in the 36-, 48- and 60-h cultures [42.3, 60.5 and 68.2 (kWh) kg^{-1}] at OTRs of 26, 49 and 85 mmol $l^{-1} h^{-1}$, resp.

3. Conclusion

The comparative evaluation of the effects of agitation speed and aeration rate on the mycelium and protein yields, as well as on the economics of their production in the three investigated fungal cultures was made possible by using OTR, the common effect of the two as the basis for comparison.

In previous work (ZETELAKI-HORVÁTH *et al.*, 1978) the best OTRs for polygalacturonase and mycelium production in *Aspergillus* culture were found to be different. The most favourable OTRs for enzyme and mycelium production proved to be different, too.

Table 7

Protein yield, and energy consumption in protein production by Rhizopus cohnii
(For cultivation conditions: see Section 1.2.)

Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})									Comparison of highest values	
	26	49	60	85	93	100	126	135	179	s	t
	Protein yield (g l^{-1})										
36	11.1	[11.9]	11.6	9.1	8.5	9.1	9.7	9.6	9.9	0.5	3.6*
48	9.6	11.9	[12.6]	9.8	9.3	9.7	9.5	10.9	10.1	0.8	0.9⁻
60	9.7	[13.1]+	10.8	11.6	10.5	11.7	11.7	8.6	9.3	0.1	0
	Protein yield produced by unit energy input [$g(kWh)^{-1}$]										
36	[23.6]+	22.1	19.0	19.2	17.0	16.9	16.9	15.7	15.4	1.6	0
48	15.3	[16.5]	15.5	15.5	13.9	13.5	12.9	13.4	11.9	3.7	3.3*
60	12.4	14.5	10.1	[14.7]	12.5	13.0	12.3	8.4	8.7	0.3	51.4***
	Energy input on the production of 1 kg mycelium (kWh)										
36	[42.3]+	45.3	52.7	52.1	58.9	59.2	59.1	63.6	65.0	3.6	0
48	65.2	[60.5]	64.7	64.3	72.0	74.2	77.3	74.8	84.2	8.9	3.5*
60	80.6	68.7	94.2	[68.2]	79.6	77.0	81.5	118.4	115.3	1.3	34.9***

[ ] highest value in the horizontal row

[ ]+ highest value in the three horizontal rows belonging to the three ages of culture

s: standard deviation
0: basis for comparison
⁻: not significant ($P < 95\%$)
*: significant ($P \geq 95\%$)
**: highly significant ($P \geq 99\%$)
***: very highly significant ($P \geq 99.9\%$)

Table 8

Conditions (time of cultivation, oxygen transfer rate, agitation and aeration) found best for mycelium and protein synthesis and energy input in the experiment carried out with the three fungi

Culture	Age of culture (h)	Oxygen transfer rate (mmol $l^{-1} h^{-1}$)	Agitation rpm.	Air consumption ($l\ min^{-1}$)	Yield ($g\ l^{-1}$) mean	s	t			
			Mycelium yield							
Actinomucor	36	60	460	1.5	22.3	2.6	10.6***			
Mucor	60	179	940	1.5	[38.5]	0.5	0			
Rhizopus	48	60	460	1.5	36.5	1.0	3.3*			
			Protein yield							
Actinomucor	48	93	940	0.5	11.3	1.5	3.9*			
Mucor	60	179	940	1.5	[14.9]	0.3	0			
Rhizopus	60	49	460	1.0	13.1	0.2	9.9***			
	Energy input on mycelial yield				$g(kWh)^{-1}$	s	t	$kWh\ kg^{-1}$	s	t
Actinomucor	36	26	460	0.5	38.1	0.9	35.7***	26.2	0.5	27.8***
Mucor	36	85	700	0.5	[57.2]	0.8	0	[17.5]	0.2	0
Rhizopus	36	26	460	0.5	56.4	0.2	3.9*	17.7	0.1	1.1⁻
			Energy input on protein yield							
Actinomucor	36	93	940	0.5	19.8	2.1	2.5⁻	50.6	3.6	3.4*
Mucor	36	26	460	0.5	22.5	1.5	0	44.3	1.8	0
Rhizopus	36	26	460	0.5	[23.6]	1.1	2.1⁻	[42.3]	2.9	1.2⁻

[ ] best value for the three strains tested
___ value next to the best

s: standard deviation
0: basis for comparison
⁻: not significant ($P < 95\%$)
*: significant ($P < 95\%$)
**: highly significant ($P \geq 99\%$)
***: very highly significant ($P \geq 99.9\%$)

Table 9

Comparison of the conditions leading to highest protein production from the points of view of highest yield and lowest energy consumption, resp.

Strain		Age of culture (h)	Oxygen transfer rate (mmol l^{-1} h^{-1})	Agitation (rpm)	Aeration rate (l min^{-1} l^{-1})	Protein yield (g l^{-1})	t value for the difference in protein yields
Actinomucor	1	48	93	940	0.5	11.3	0.5–
	2	36	93	940	0.5	10.9	
Mucor	1	60	179	940	1.5	14.9	21.8***
	2	36	26	460	0.5	9.8	
Rhizopus	1	60	49	460	1.0	13.1	3.6*
	2	36	26	460	0.5	11.9	

1 highest protein yield
2 lowest energy consumption

– not significant ($P < 95\%$)
* significant ($P \geq 95\%$)
*** very highly significant ($P \geq 99.9\%$)

In the present work, the most favourable parameters for protein and mycelium yields were identical only in the case of the *Mucor* strain. The parameters of the highest mycelium yield (g l^{-1}) and the most economic production (highest quantity of mycelium produced by 1 kWh) were different for all three strains tested (Table 8).

The economically most favourable conditions for mycelium and protein production also proved to be different. Only the most advantageous time of cultivation was the same in both cases, namely the 36 h.

The mycelium and protein yields of the *Mucor* culture were very highly significantly and significantly higher than those of the *Actinomucor* culture and significantly and very highly significantly higher than those of the *Rhizopus* culture, resp.

The energy consumed for the production of 1 kg mycelium or protein was very highly significantly lower in the *Mucor* culture than in *Actinomucor* culture, while no significant differences were found between the energy consumptions of the *Mucor* and the *Rhizopus* cultures.

When the protein yields of these strains were compared under conditions producing the highest protein yields and resulting in the lowest energy consumption (Table 9), no significant differences were found between the two protein yields in the *Actinomucor* culture, while in the *Rhizopus* and the *Mucor* cultures significant and very highly significant differences could be established.

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COMPUTER EVALUATION OF THE RESULTS OF BATCH FERMENTATIONS

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A useful aid to the mathematical modelling of fermentation systems, to the kinetic evaluation of batch fermentations, is described. The generalized logistic equation, as suggested, may be used to describe the growth curves, substrate consumption and product formation. A computer process was developed to fit the equation to experimental points, automatically determining the equation constants on the basis of the iteration algorithm of the method of non-linear least squares. By fitting the process to different master programmes of various fermentations, the complex kinetic evaluation of fermentations becomes possible. Based on the analytically easily treatable generalized logistic equation, it is possible to calculate by computer different kinetic characteristics: *e.g.* rates, specific rates, yields, *etc.* The possibility of committing subjective errors, so common in kinetic evaluation, was reduced to a minimum. Employment of the method is demonstrated on some fermentation processes and problems arising in the course of application are discussed.

A precondition of the realization of large-scale industrial fermentation technologies is pilot plant scaling-up research. In this phase of fermentation research these are necessary steps and the requirement is, beyond the traditional qualitative view, more and more the quantitative knowledge. In this process of quantitative cognition mathematics are an important aid in mathematical modelling of the growth and synthesizing activity of microorganisms and its kinetic description.

Of the kinetic models of batch fermentations some are widely employed. One of these is the well-known MONOD model or family of models, or the KONO-ASAI model, also well-known. A critical analysis of models suitable for application in batch fermentations was given in a summarizing study of the authors (HOLLÓ *et al.*, 1978). An auxiliary model – the generalized logistic equation – is discussed in detail in the present paper with special reference to problems of applicability.

1. Some problems of mathematical modelling

The results of batch fermentation experiments are most frequently c_i–t pairs of points, thus, cell concentration, substrate concentration, product concentration are given as functions of time while mathematical models describing fermentation systems are mostly of differential equation character.

$$\frac{dc_i}{dt} = f_i(c_1, c_2 \ldots c_n, a_0 \ldots a_m, t)$$

where f stands for a function relation
t is the fermentation time
c_i is the concentration of the i-th component of the system
a parameters of the model to be determined.

Even if the differential equation system of the model may be solved in a form which can be directly fitted to the experimental results, it is frequently necessary to know, already in the stage of devising the model, the velocities of the quantities characterizing the system.

The problem arises of how the experimentally obtained c_i vs. t curve, known only in points, should be differentiated? Generally the method: "idealization of the curve" is employed, *i.e.* the points measured are connected by a continuous curve and then the curve is graphically differentiated. It is beyond doubt that a limitless number of idealized curves may be plotted between c_i–t pairs of points (it is generally characteristic of fermentations that during a relatively long period only a few points of measurement can be obtained).

Thus, to the relatively great errors of experimental results (these may reach even 10 % in the case of biological measurements, indirect determinations of cell density) a fresh and moreover subjective error is added, heaped in addition with another source of error, graphical differentiation.

Graphical differentiation and the idealization of curve may be eliminated by the use of an auxiliary model, which

- is an analytically treatable function of closed formula,
- continuous and continuously differentiable,
- has not too many parameters to be determined (at least some of which have a physical sense),
- comprehends in a single formula the whole experimental range of batch fermentations,
- independently of given experimental conditions is suitable to describe every kind of batch fermentation.

A polynomial of higher order apparently satisfies the requirements, however, in the case of a low number of degrees (2–3) this is not sufficiently flexible, at a higher number of degrees, on the other hand, the inflection points

and local limit values are, with increasing number of degrees, less and less suitable for the given purpose.

The generalized logistic equation proved to be a suitable and generally employable auxiliary model. This was first used to describe microbial growth by EDWARDS and WILKE (1968). The generalized logistic equation is an extension of the logistic equation satisfactorily describing problems of various kinds (filtration, multiplication). The extension means further time dependence of one of its parameters. The generalized formula is as follows:

$$y = \frac{y_{max}}{1 + \exp(a_0 + a_1 t + \ldots + a_n t^n)} \tag{1}$$

In some earlier studies of the authors (SEVELLA, 1975; HOLLÓ *et al.*, 1978), it was proved that equation (1) is applicable not only to the growth of the population, but to substrate consumption and product formation, too.

2. Use of the auxiliary model to the kinetic evaluation of batch fermentations

The generalized logistic equation is expedient to be used as auxiliary model in order to avoid graphical differentiations needed for kinetic evaluations. The expression *auxiliary model* is stressed because its use is a device promoting the setting up and control of a so-called *work model*, describing more adequately the essence of the system. Since it is suitable to describe not only growth, but other important time functions of batch fermentations, it enables their complex kinetic evaluation.

The fitting of the auxiliary model to the experimental results means the determination of the parameters y_{max}, $a_0 \ldots a_n$ of equation (1), beside which the error sum of squares $\Sigma (y_{i\,calculated} - y_{i\,measured})^2$ is minimal. By the use of the method of non-linear least squares (SCARBOROUGH, 1962) the development of a computer process became possible, by which the parameters are automatically identified. The integration of this process in different master programmes provides the possibility of the complex kinetic evaluation of different fermentations. Namely, after the calculation of the constants it becomes possible to calculate by the equations any kinetic characteristic (rates, specific rates or accelerations, *etc.*) at any time. The fitting programme and the various master programmes were written in ALGOL-60 language (as special representative of ODRA-1204 computer). Their use in kinetic analysis is demonstrated in relation to various fermentations.

2.1. Kinetic analysis of sorbose fermentation

The aim of the experiments was the study of the sorbitol → sorbose conversion catalyzed by *Acetobacter suboxydans* ATCC 621 strain in the batch fermentation system of growing cultures. Description of experimental condi-

Table 1

Data of batch sorbose fermentations
(NYESTE, 1965)

Fermentation time (h)	Optical density (OD_{540})	Sorbose (mg cm^{-3})
0	0.056	0.60
1.5	0.064	0.77
3.0	0.085	1.50
4.0	0.117	2.10
5.0	0.242	2.65
6.0	0.360	4.65
7.0	0.530	8.50
8.0	0.895	15.30
9.0	1.35	25.60
10.0	2.23	39.20
11.0	3.21	50.70
12.0	4.05	64.50
13.0	5.01	75.20
14.0	5.64	87.80
15.0	5.82	90.30
16.0	6.02	90.30

tions and analytical methods is disregarded here (NYESTE, 1965). A typical experimental record is shown in Table 1.

In Fig. 1 the points as measured and as calculated, on the basis of generalized logistic equation, by computer, are illustrated. The results fit extremely well both for the growth and the product formation curve. These are numerically expressed in the statistical characteristics calculated by computer and shown in Table 2.

On the basis of the above it may be said that by means of the generalized logistic equation it is possible to tabulate the points calculated at any time interval and thereby substitute idealization of the curve. The actual advantage of the method lies in the calculability of the rate values and that they can be tabulated (as a nearly continuous curve) at any point of time. In Fig. 2

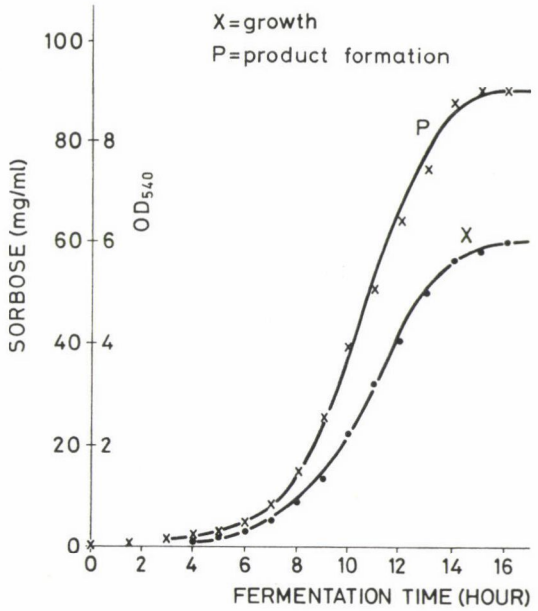

Fig. 1. Fitting of the generalized logistic equation to experimental results of sorbose fermentation: kinetic representation of the fermentation. •——• growth; ×——× product formation

Table 2

Statistical characteristics of the generalized logistic equation fitted to data of growth and product formation in sorbose fermentation

	Standard deviation about the regression curve	Correlation coefficient
Growth	0.002412 (OD)	0.999781
Product formation	0.30742 (mg cm^{-3})	0.999878

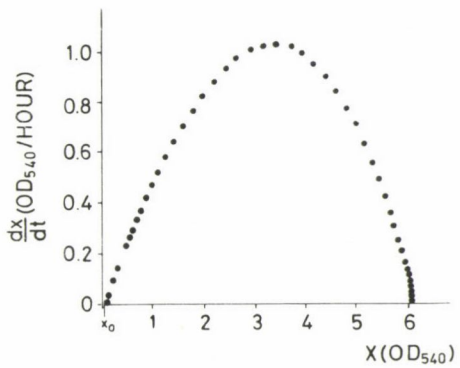

Fig. 2. Growth and growth rate curves of sorbose fermentation

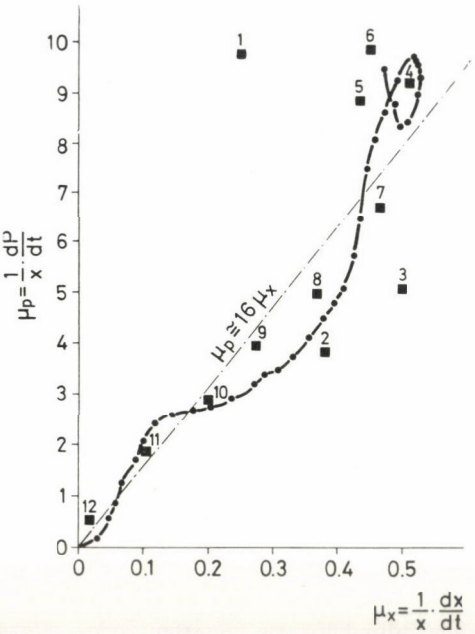

Fig. 3. Relationship of growth and product formation in sorbose fermentation. □ 1, □ 2, . . . □ 12 points obtained by manual calculation with increasing numbers in order of passing time; — · — · — · —: regression line fitted to points obtained by manual calculation; — • — • —: values calculated by computer with the auxiliary model

dx/dt vs. x, customary in the kinetic evaluation of batch fermentations, is shown. This may be plotted to any degree of continuity. Thus, designing chemostat type continuous fermentations on the basis of batch results is substantially more accurate than non-computerized calculations.

A further advantage of the auxiliary model is shown in Fig. 3.

In this figure the values of specific product formation rates are plotted against values of specific growth rates calculated by hand and by computer. The former points may be used in evaluating according to LUEDEKING and PIRET (1959) the product formation kinetics. The points may be connected by a line starting from the origin and having a slope of about 16. If, however, the order of points in time is also taken into account some sort of tendency of advance may be derived even from the points calculated by hand. The situation becomes much clearer if results of computer calculations are considered. Here the pattern of points is not scattered but closely dependent on fermentation time. The example shown points to the fact that kinetic analysis based on curve idealization by hand and subsequent graphical differentiation, is strongly subjective – the more so the more complex graphical interpretation is needed – and may lead to a result completely concealing actual relation-

ships. In this case, for instance, it would be misleading to conclude that sorbose fermentation goes with 1st type product formation, *i.e.* product formation depends only on growth. It has been experimentally proved (fermentation of resting cells) that also non-growing cells participate in product formation.

2.2. Kinetic analysis of gluconic acid fermentation

Acetobacter suboxydans ATCC 621 is capable not only of the sorbitol → sorbose conversion. It can oxidize a number of sugars, sugar alcohols and glycerol as well. Thus, it is capable of converting glucose in a consecutive series of reactions into gluconic acid, finally into 5-keto-gluconic acid (through glucono-δ-lactone) under both, resting cell and growing cell conditions (SZŐKE, 1978; SZŐKE *et al.*, 1974). To follow up growth and glucose conversion under fermentation conditions, at least 4–5 parameters have to be measured simultaneously. In the course of these experiments the following parameters were measured:*

- cell density,
- glucose concentration,
- concentration of reducing groups,
- alkali used for neutralizing the acid formed,
- occasionally: concentration of intermediary lactone.

In the course of a fermentation with sampling every half or full hour, only about 14–15 measurements were available for kinetic analysis. Evaluation by hand – even if the simplest requirements were satisfied: growth rate, substrate consumption and product formation were calculated – was a task taking several days, and as shown above, not devoid of subjective error.

Using the auxiliary model, as suggested, complete evaluation takes only a few hours of which the run of the computer programme lasts some minutes the rest of the time being taken by data preparation and by analysis of results. A complete kinetic analysis is understood to mean: fitting the generalized logistic equation to all variables characteristic of the fermentation, recording at arbitrarily chosen intervals the calculated values and calculation and tabulation of rates and yields of interest. These were as follows:

I. Primary kinetic quantities (values calculated from the generalized logistic equation fitted to the measured values):

1: cell density, x
2: glucose concentration, g
3: reducing substance content (glucose + 5-keto-gluconic acid), r

* Gluconic acid fermentation, related biochemical problems and the methods of measurement, employed in this paper are discussed in detail by SZŐKE (1978) and the kinetic modelling of gluconic acid fermentation by SEVELLA (1975), and NYESTE and co-workers (1979).

4: concentration of 5-keto-gluconic acid, c
5: glucono-δ-lactone concentration, l
6: consumed alkali (to keep pH at a constant level), al
7: concentration of gluconic acid formed, ga

II. Rates

8: dx/dt growth rate
9: dg/dt rate of glucose (substrate) consumption
10: dc/dt rate of 5-keto-gluconic acid formation
11: dl/dt rate of change in lactone concentration (formation–consumption rate)
12: dal/dt rate of alkali consumption (change of pH)
13: dga/dt rate of change in gluconic acid concentration (formation–consumption rate)

III. Specific velocity values

14: μ_x specific growth rate
15: μ_g specific rate of glucose consumption
16: μ_{ga} specific rate of gluconic acid formation
17: μ_c specific rate of 5-keto-gluconic acid formation
18: μ_{al} specific rate of alkali consumption (change of pH)

IV. Expressions of yield character

19: dx/dg;
20: dga/dg;
21: dc/dg:
22: dc/dga;
23: dga/dx;
24: dc/dx;
25: dal/dg

V. Reciprocals of values in groups I and III: 26–38.

The 38 data as listed above are not necessarily needed, but on the other hand it is true that further combinations of data (*e.g.* product of various members of groups I and II, various sums, *etc.*) may be formed if these are required in accordance with the conception of the modeller. Evidently, in this case the logistic equation, as an auxiliary model, is a help in developing the work model.

2.3. Some problems related to the suggested auxiliary model

It is evident from the form of equation (1) that a choice is presented to the user between equations containing polynomials of different degrees.

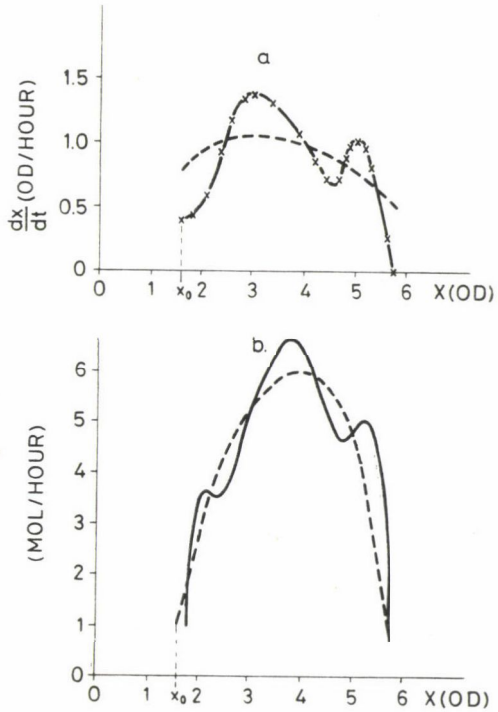

Fig. 4. Kinetic illustration of gluconic acid fermentations. *a)* Growth rate as a function of growth; *b)* gluconic acid production rate versus growth. Comparison of curves calculated by generalized logistic equation containing 5th degree polynomial (———) and by that containing 3rd degree polynomial (– – – –), resp.

For the kinetic evaluation of batch fermentations the generalized logistic equation, containing polynomials of the 1st, 3rd and 5th degree, is applicable. In the case of sorbose fermentation, the equation has to be fitted to two, and in gluconic acid fermentation to five series of data, thus, in the first case 8, in the latter 125 different ways of running the master programme were possible. The problem arises as to which degree of polynomial should be applied. The knowledge of the properties of the generalized logistic equation shows that the flexibility of the equation increases with increasing degree of the polynomial applied. Thus, in the case of using a 5th degree polynomial, the auxiliary model is suitable to describe diauxic growth. Increased flexibility, however, increases the probability of drawing erroneous conclusions. This case is shown in Fig. 4, where the curve of gluconic acid fermentation rate was illustrated based on two different polynomial degrees.

The curves both for growth and for product formation have two or three peaks on applying a 5th degree polynomial, however, this is not indicated in case of this fermentation. This phenomenon occurs only in case of diauxic

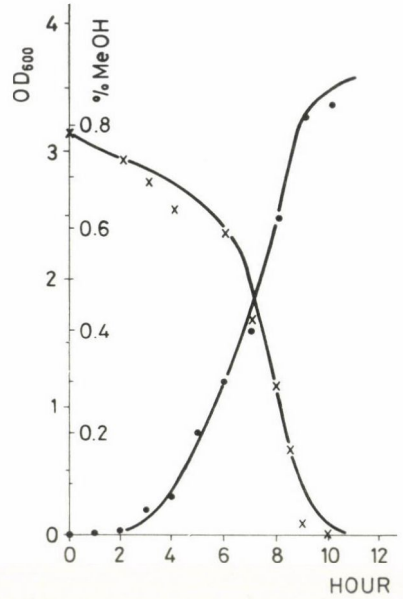

Fig. 5. Kinetic representation of SCP (Single Cell Protein) fermentation on methanol, measured values and calculated curves (KIRCHKNOPF, 1979). ×– – –×: methanol consumption; o– – –o: growth

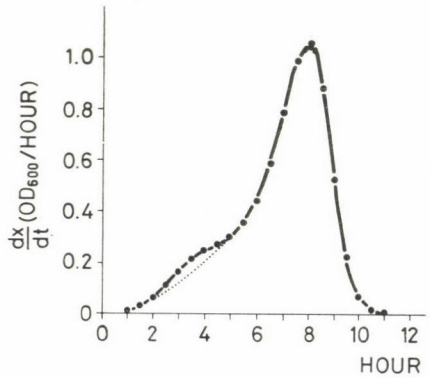

Fig. 6. Growth versus growth rate curve of SCP fermentation on methanol. — · — · —: values calculated by computer; · · · · · · · : probable shape of curve

growth or of environmental change, *e.g.* error in temperature control. None of these factors occurred in the given gluconic acid fermentation. Both rate curves are smoothed out when a polynomial of third degree is used, thus the drawing of a wrong conclusion is excluded.

The phenomenon shown (appearence of local extreme value on the rate curves, their prevalence on primary curves fitted to experimental points) is caused by unexpected or highly scattered points, when the fitted curve with higher polynomial degrees connects the outstanding points, too. In the case shown, with lower polynomial degrees, the points are automatically smoothed out by fitting.

There is no general rule of the polynomial degree to be employed, however, the following observations may help with great probability:

– In the case of highly scattered points it is expedient to use the form of equation containing polynomials of 1st or 3rd degree.

– Good fitting may be expected if the number of experimental data exceeds several times the degree of polynomial applied. However, in doubtful cases it is advisable to investigate fittings run in different ways in order to avoid erroneous information.

A related negative example is shown in Figs. 5 and 6.

Figure 5 shows a growth curve of good fitting. In Fig. 6 it is striking that in the interval between the 2nd and 5th hours of the experiment two more inflections are to be found on the rate curve, which is under the known experimental conditions alltogether improbable, thus it does not follow from the behaviour of the microorganism, it is the consequence of fitting. This is the more so as the phenomenon does not appear on the growth curve (Fig. 5, solid line). Thus, the 3rd degree polynomial was logically chosen: relatively scattered points, data are more numerous than the polynomials. However, in spite of good fitting it would have been more expedient to use a 1st degree polynomial.

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TREATMENT OF WASTE WATERS CONTAINING PROTEIN AND CARBOHYDRATES USING ACTIVATED CELLULOSE AND LIGNITE

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Removal of the pollutants from liquid wastes formed in the food industries is difficult because they differ in quantity and composition in each branch. A common feature is, however, that they contain, besides other substances, proteins and carbohydrates. In order to reduce these they were subjected to treatment with polyelectrolytes, inorganic salts, activated cellulose and lignite derivatives. The polyelectrolytes *Combifloc* and *Praestol B 714* proved efficient only in waste waters of industrial origin, causing a reduction higher than 20.0 ± 2.1% in the COD.

The result, measured by the reduction in cations, was achieved in alkaline waste waters of meat and sugar industrial character by using the inorganic coagulant $Al_2(SO_4)_3$. Inorganic salts, not very effective in neutral media, may be improved by combined use. A reduction of 66 ± 31% may be achieved *e.g.* by combining $MgCl_2$ and $CaCl_2$. A further 10% reduction was achieved by the threefold combination of $Fe_2(SO_4)_3 + MgCl_2 + Al_2(SO_4)_3$.

Cellulose activated with inorganic salts and lignites treated with sulfuric acid are also effective in reducing the organic content of meat processing effluents by 50 ± 31% and ±19.5%, resp.

Hygienic requirements in food processing plants necessitate the possibly early removal of the waste materials produced. The first step towards this aim is to remove the particulate matter by means of appropriate screens and settling equipment (HERSICZKY & KIRÁLY, 1972; BARTHA *et al.*, 1976; LÁSZTITY & TÓTH, 1978). However, the equipment and technologies available have been designed for the treatment of communal sewage (HUSMANN, 1973).

One of the difficulties in the treatment of waste waters formed in the food industries is that their composition and quantity varies between and within branches. Shortly after their formation they may start decaying and developing offensive odour. Thus, every attempt to remove the wastes as early as possible is conducive to maintaining appropriate hygienic conditions in the plant. The aim of the present experiments was to improve hygienic conditions by the treatment of waste waters formed in the meat, starch and sugar industries. Beside the waste waters, model substances were also used in the experiments.

Among the foregoing branches of the food industry the worst conditions prevail in the meat industry, therefore, in the relevant experiments all available materials were tested. The waste waters from sugar refineries were also sub-

jected to more detailed investigation, since owing to the high water demand of this industry, it is important to reduce the organic matter in the primary effluent. The waste waters of corn starch manufacture were studied because protein and carbohydrates are simultaneously present in them. In this case preliminary experiments promised the reduction of organic matter by the application of polyelectrolytes.

1. Materials and methods

1.1. Materials

1.1.1. Organic and inorganic coagulants. Of the organic coagulants the polyelectrolytes trademarked *Praestol B 714* and *Combifloc* were tested in 1% solutions.

The inorganic coagulants traditionally used in waste water treatment: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$ and $CaCl_2$, were tested individually and in different combinations. The inorganic coagulants of analytical grade were used in 10% solution in the proportion of 5 ml to 0.5 l waste water. After the addition of the reagents the mixture was blended for 1 min at 60 rpm.

1.1.2. Fibrous and solid surface active agents. In view of the utilization of sludges formed in the course of waste water treatment two new agents were tested.

In order to use the sludge as cattle feed, brown and bleached cellulose were used. These were pulped and activated with inorganic coagulants prior to use.

To develop a type of sludge for incineration, ground lignite was tested in the raw state and after drying. To activate the lignite, 50% analytical grade sulfuric acid solution was applied.

1.1.3. Model and industrial waste waters. Model solutions, based on data in the literature, were prepared as follows:

Meat industry (HORVÁTH, 1972)

0.100 g cm^{-3}	meat broth
0.150 g cm^{-3}	pepton
0.026 g cm^{-3}	urea
0.006 g cm^{-3}	NaCl
0.002 g cm^{-3}	$MgCl_2 6 H_2O$
0.004 g cm^{-3}	$CaCl_2 2 H_2O$

Sugar industry (SZABÓ, 1975)

0.05 g l^{-1}	beet sugar
1.00 g l^{-1}	kaolin

0.01 g l^{-1}	white of egg
0.03 g l^{-1}	NaCl
0.017 g l^{-1}	CaO
0.10 cm^3 l^{-1}	1 N H_2SO_4

Waste waters of industrial origin were obtained from the outlet of the PIG SLAUGHTERHOUSE of the BUDAPEST MEAT INDUSTRY from the flume water of the MÁTRA SUGAR REFINERY and from the ÁSZÁR CORN STARCH FACTORY, resp.

1.2. Methods

1.2.1. Determination of the residual cation content in the inorganic coagulants. Changes in the concentration of inorganic substances were traced by the determination of their cations. The extent of removal was estimated from the reduction in concentration after a contact period of 30 min.

To determine the Al content in the waste waters by *Daubner's* method a volume corresponding to about 10 mg aluminium was removed. This was slightly acidified with acetic acid and the aluminium precipitated in the presence of NH_4Cl with $(NH_4)_3AsO_4$ as $AlAsO_4$. The precipitate was filtered, washed and dissolved in hydrochloric acid and iodometrically titrated. 1 cm^3 of 0.1 N $Na_2S_2O_3$ corresponds to 1.3485 mg Al (ERDEY, 1966).

The residual Fe^{3+} ions were determined by complexometry. To the slightly acid solution NaOH was added till precipitation and the precipitate was dissolved by adding formic acid. Once the precipitate was dissolved, the solution was heated to 40–50 °C, 1 drop of *Variamine-blue B* indicator was added and it was titrated with *Complexon* III solution. Titration was finished when the colour corresponding to that of ferric complexonate turned pure yellow. 1 cm^3 of 1 M *Complexon* III solution corresponds to 0.5585 mg Fe (ERDEY, 1966).

Complexometry was applied to determine Mg and Ca, too. Ca was determined directly with murexide indicator. To find the Mg concentration, the number of cm^3 used was detracted from the *Complexon* III used for the joint determination of both ions. In the latter titration *Eriochrome-black T* indicator was used. 1 cm^3 of *Complexon* III is equivalent to 0.4008 mg Ca, or 0.2432 mg Mg (ERDEY, 1966).

1.2.2. Use of inorganic coagulants. The inorganic salts: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$, $CaCl_2$, were used individually or added in sequence with 30 s intervals, in a proportion of 1 g to 1 l. The effect of the salt was established after agitation for 1 min at 60 rpm and 30 min resting period.

1.2.3. Determination of the chemical oxygen demand. To measure the chemical oxygen demand (COD) of waste waters the potassium dichromate

method was used as described in HUNGARIAN STANDARD (1968). The organic matter in waste waters is most radically degraded by this method and made available to oxidation by dichromate. Generally, a sample of 20 cm^3 is used or proportionately less from more heavily polluted effluents. These latter are made up with distilled water to 20 cm^3. Then the 0.25 N potassium dichromate solution is added and 0.4 g mercury sulfate and finally 30 cm^3 of sulfuric acid containing silver sulfate. The mixture is then boiled for 1 h under reflux. The amount of dichromate used up is established by titration with ammonium sulfate containing 0.25 N Fe II.

2. Results

2.1. Experiments with polyelectrolytes

The effect of organic coagulants was tested in model solutions, as well as in the waste waters of starch manufacture containing both protein and carbohydrates simultaneously.

The COD of the model solution simulating waste water of the meat industry was originally of 208 ± 17 mg l^{-1}. This value was reduced by *Combifloc* to 177 ± 65, while *Praestol B-714* was completely ineffective. The difference between the original and treated model solutions simulating waste waters of the meat industry was significant only between the probability levels of $P = 80$ and $P = 90\%$.

Fluctuation of this extent was not observed in model solutions simulating waste waters of the sugar industry. The COD of the upper phase was reduced by treatment with *Combifloc* from 770 ± 24 to 565 ± 78 mg l^{-1}. A somewhat better result was achieved with *Praestol* with a reduction to 550 ± 18 mg l^{-1}. These results showed a significant difference by t test at the probability level of $P = 99.9\%$.

The effect of polyelectrolytes was tested with two kinds of waste water of the starch industry. The combined plant effluent contained, in addition to suspended starch granules, dissolved carbohydrates and protein, as well as wastes of syrup and dextrose manufacture (activated carbon, *kieselguhr, etc.*). The COD of this effluent was found in 5 parallel determinations to be $2450 \pm \pm 284$ mg l^{-1}. This was reduced by the use of *Combifloc* to 1855 ± 193 and by that of *Praestol* to 1950 ± 270. In comparison with the original value the effect of both polyelectrolytes was significant at the probability level of $P = 99.9\%$.

The second kind of waste water was that from the starch factory only. The husks and corn protein were removed therefrom by settling. (The former effluent contained this phase as well.) Since settling does not affect the dissolved substances, the COD remained significant. The average value obtained in the analysis of 5 separate samples was found to be 2880 ± 341 mg l^{-1}. This

Table 1

Effect of Combifloc *and* Praestol B 714 *polyelectrolytes on model waste waters of the sugar and meat industries and industrial waste water of the corn starch industry*

Serial number	Sample of waste water	Organic removal in the upper phase after 30 min contact period	
		Combifloc	*Praestol*
1	Model waste water of the meat industry	15.0%	–
2	Model waste water of the sugar industry	17.0%	18.5%
3	Total effluent of the corn starch factory	24.0%	20.0%
4	Waste water of the starch factory after sedimentation	10.0%	8.0%

value was reduced by *Combifloc* to 2607 $\pm$ 354 and by *Praestol* to 2650 $\pm$ 273 mg l^{-1}. These changes were, however, significant only at the $P \geq 90$–95% level.

The effect of the various treatments calculated as percentages are shown in Table 1. The Table shows the differences between the COD of the original waste water and that in the upper phase of the treated water, 30 min after treatment.

2.2. *Inorganic coagulants*

The effect of the individual inorganic coagulants was studied in waste waters of the meat and sugar industries. Based on earlier experiences, the experiments were carried out in alkaline media (HERSICZKY & KIRÁLY, 1972). In order to gain a better insight into the mechanism instead of the change in COD, the reduction in the concentration of the individual cations was determined in the upper phase, after contact periods of 30 min. It was presumed that the reduction of the cations occurred by their precipitation and descent to the lower phase.

The coagulants were applied to the waste waters in 1 g l^{-1} concentration. The results in Figs. 1, 2, 3 and 4 are the averages of 10 parallel measurements each. Deviations did not exceed $\pm 1.95\%$, therefore these were not illustrated. The differences between the original concentrations and the measured values after treatment are significant even at the highest level of probability.

As to be seen in the Figures, except for $MgCl_2$, the inorganic coagulants are very effective in clarifying the upper phase. However, this effect is highly pH dependent.

The effect of the inorganic coagulants was tested also by their combinations. In these experiments only waste waters of the meat industry were used and the pH was not modified. It was assumed that the good results achieved

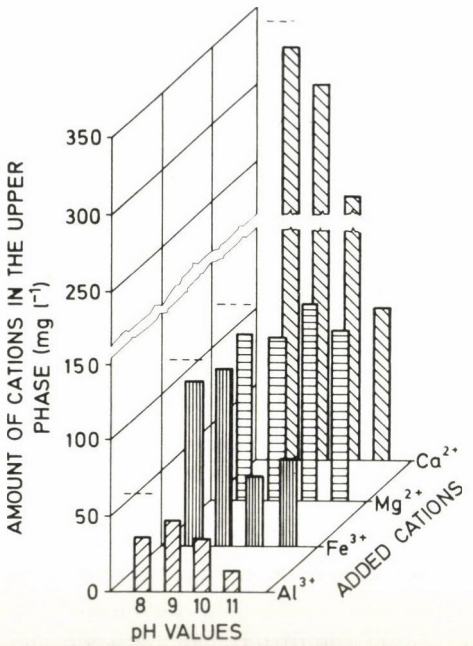

Fig. 1. Cation content in the model waste water of the meat industry on adding $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$ and $CaCl_2$, resp., and after a resting period of 30 min, *vs.* pH, in the upper phase of the water. (– – – –) the dashed line marks the ion concentration at 0 time

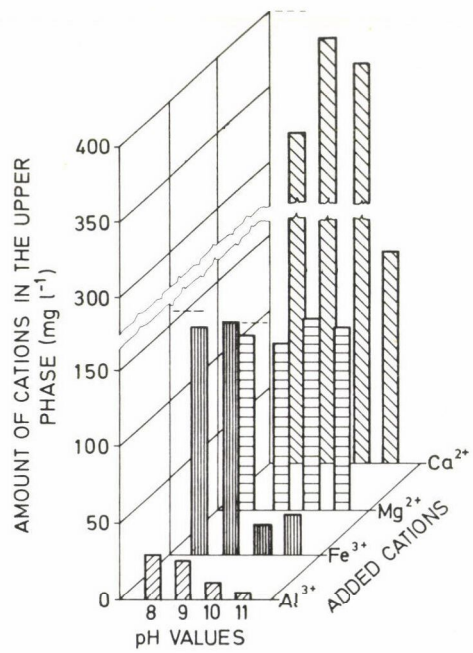

Fig. 2. Cation content in the waste water of the meat industry on adding $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$ and CaCl, resp., and after 30 min resting period, *vs.* pH, in the upper phase of the water. (– – – –) the dashed line marks the ion concentration at 0 time

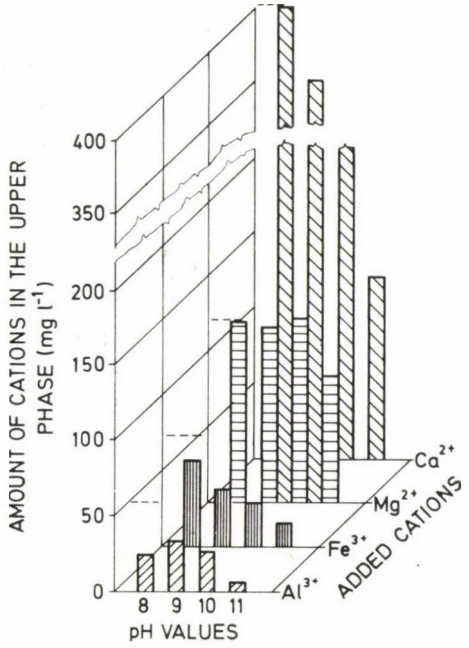

Fig. 3. Cation content in model waste waters of the sugar industry on adding $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, MgCl and $CaCl_2$, resp., and after 30 min resting period *vs.* pH, in the upper phase of the water. (– – – –) the dashed line marks the ion concentration at 0 time

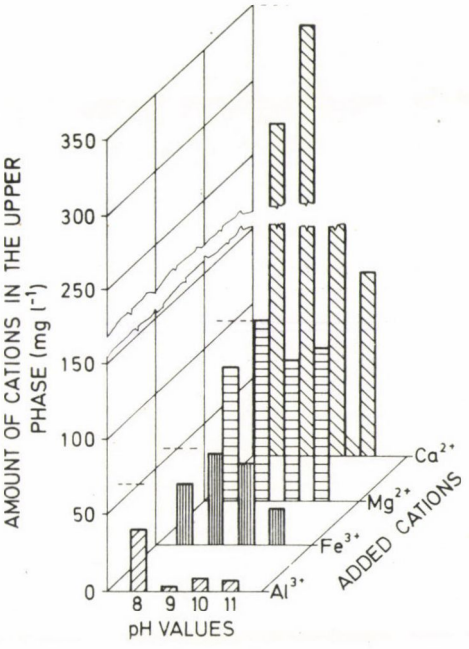

Fig. 4. Cation content in the upper phase of the sugarbeet flume water upon adding $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$ and $CaCl_2$ *vs.* pH. (– – – –) the dashed line marks the ion concentration at 0 time

occasionally in media of adverse pH from the point-of-view of coagulants may be increased by further lowering the pH. Since the simultaneous detection of cations was difficult, the removal effect was again measured by the reduction in COD. The averages of 10 parallel experiments are given in Table 2 and 3.

The deviations from the results obtained by treatment with polyelectrolytes in model solutions simulating waste water of the meat industry were insignificant (max. 2.11%). The difference between the original and treated solutions is significant at the level of $P = 99.9\%$.

The more closely approximate actual conditions, the results shown in Table 3 were not derived from a single sample, as done earlier, but from tests on samples taken at different occasions. The samples were taken nine times at 48-h intervals and the average COD was 2239 ± 683 mg l^{-1}. Deviations

Table 2

Treatment of the model simulating waste waters of the meat industry by the combined use of two inorganic coagulants at pH = 7

Ions added second	Percentage reduction of the COD by inorganic ions			
	Ions added first			
	Al	Fe	Mg	Ca
Al	–	6	35	4
Fe	15	–	22	15
Mg	17	20	–	0
Ca	12	30	19	–

The cations were added in the form of the following compounds: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$, $CaCl_2$ in a concentration of 1 g l^{-1}

Table 3

Treatment of the waste waters of the meat industry by the combination of two inorganic coagulants at pH = 7

Ions added second	Percentage reduction of the COD by inorganic cations			
	Ions added first			
	Al	Fe	Mg	Ca
Al	–	46	41	50
Fe	56	–	35	52
Mg	47	49	–	30
Ca	60	66	38	–

The cations were added in the form of the following compounds: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$, $CaCl_2$ in a concentration of 1 g l^{-1}

Table 4

Treatment of the waste waters of the meat industry by the combination of three inorganic coagulants at pH = 7

Ions added second and third	Percentage reduction of the COD by inorganic coagulants			
	Ions added first			
	Al	Fe	Mg	Ca
Al–Ca	–	38	–	–
Al–Fe	–	–	–	32
Fe–Ca	31	–	–	–
Fe–Al	–	–	–	44
Fe–Al	–	–	38	–
Fe–Mg	37	–	–	–
Mg–Al	–	45	–	–
Ca–Al	–	26	–	–
Ca–Fe	34	–	–	–

The cations were added in the form of the following compounds: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $MgCl_2$, $CaCl_2$ in the concentration of 1 g l^{-1}

remained unaltered after treatment with the inorganic reagents. Differences were found significant by the t test at the probability level of $P \geq 99.9\%$.

Comparison of Tables 2 and 3 show that the combined application of inorganic salts is a more effective means of industrial waste water treatment.

Finally the effect of treatment with three components was also studied in the order and combinations as shown in Table 4.

The standard deviations found between the ten parallel experiments did not exceed 1.47%. The application of the method appears to be justified by the fact that *e.g.* in the model simulating meat industry effluent the removal effect was highly improved by three-fold combination as compared to the single agent or a two-fold combination. The difference over the original value was found in every case to be significant at $P \geq 99.9\%$.

2.3. Effect of activated cellulose and of lignite derivatives on meat industry effluents

With the aim of reducing organic contentand improving settling behaviour two new clarifying agents were subjected to trial in preliminary studies on the treatment of effluents and sludges from the food industry. Since the two agents were not effective in their original state, they were activated. First the raw and bleached cellulose were treated with a 10% solution of the inorganic coagulants applied in the previous section, for 2 h at 100 °C. After decanting the superfluous solution from the 20 g l^{-1} cellulose suspension the residue was dried to constant weight at 105 °C. Five g of the cellulose thus activated was

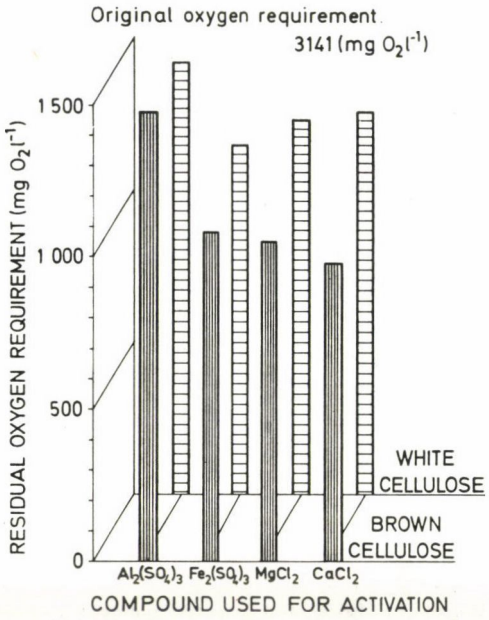

Fig. 5. The COD of waste waters of the meat industry as affected by activated cellulose after 30 min sedimentation at pH = 7

applied to each waste water sample. The sample was then agitated for 1 min and analysed after 30-min contact period. The average results of the analysis carried out at 2-day intervals throughout a month are shown in Fig. 5.

Although with different efficiencies, the cellulose samples treated with different inorganic salts caused even in the case of the least effective $Al_2(SO_4)_3$ an important reduction in COD ($P \geq 99.9\%$). The difference between the effect of cellulose treated with different agents was significant at the same level only in the case of brown cellulose treated with $Al_2(SO_4)_3$ on the one hand and $Fe_2(SO_4)_3$ on the other. The difference with white cellulose between the two treatments was significant only at the $P \geq 98$–99% level. The relative differences between the other columns in Fig. 5 do not reach the 70% probability level.

The COD values of waste waters produced in the slaughterhouse on different days varied widely, up to $\pm 45\%$, about the mean. In the upper phase of the treated effluent the difference from the monthly average amounted to $\pm 31\%$, only. Nevertheless, since the differences are not significant, the effects of the activating agents can be characterized by average values only.

The effect of the lignites in their original state was also unimportant. They were activated under laboratory conditions in a way resembling carbon activation (GOTTLIEB, 1968). The lignite was treated in 50% sulfuric acid solution at 600 g l^{-1} concentration for 1 h at 100 °C. Two different kinds of

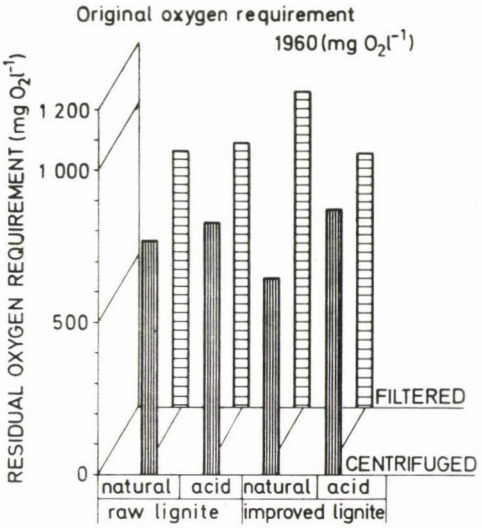

Fig. 6. The COD of waste waters of the meat industry as affected by activated raw and improved lignites

lignite were prepared from each sample. In one case the surface of lignite granules was washed with distilled water to neutral pH. In the other case the superfluous liquid was decanted and the residue carefully dried at 80 °C. The treated lignite was applied in the concentration of 5 g to 1 l waste water. The mixture was stirred for 1 min and allowed to stand for 30 min. To avoid the inclusion of lignite particles remaining in suspension, the upper phase was not only decanted, but also filtered or centrifuged. The results are shown in Fig. 6.

Similarly to cellulose, the various lignite samples also reduced the COD ($P \geq 99.9\%$). Of the samples washed after activation, the centrifuged raw lignites differed significantly at the 90–95% probability level from the original ones. The difference between the dried, or improved lignite samples was significant at the probability level of $P \geq 99.9\%$, but was of opposite sign, showing a change in the specific weight relations.

In this 1 month test period the mean and standard deviation of the samples taken every 48 h was lower than those described in the foregoing section. The COD values on individual days ranged by $\pm 28\%$ about the mean of 1968 mg l^{-1}. This range diminished to $\pm 19.5\%$ after treatment.

3. Conclusions

These preliminary experiments have shown that the organic content of waste waters containing carbohydrate and protein can be reduced substantially by adding inorganic coagulants, further cellulose and lignite derivatives.

Combifloc and *Praestol B 714* polyelectrolytes were also tested and found to reduce the organic content by 20.0 ± 2.1% or more, in the combined effluent of the starch manufacturing plant containing suspended starch granules as well. Removel in this case is probably due to the interaction of the polyelectrolytes and the suspended substances (BURR *et al.*, 1975). In the model simulating a sugar refinery effluent, the kaolin seemed to reduce the COD, only to a smaller extent.

Inorganic coagulants proved substantially more effective in an alkaline medium. In waste waters of the meat industry $Al_2(SO_4)_3$ proved superior to $Fe_2(SO_4)_3$, MgCl and $CaCl_2$. The same applies to sugar refinery effluents, only with model waters the best result was achieved at pH = 11, while with industrial waters at pH = 9. Besides $Al_2(SO_4)_3$, also $Fe_2(SO_4)_3$ was found to reduce the cation content of the upper phase and the effluent organic matter successfully. In both cases the best result was achieved at pH = 11. In the model the reduction amounted to 75.5 ± 1.18%, in the flume water to 59.5 ± ± 1.95%. In agreement with earlier experiments, Ca^{2+} proved most effective at pH = 11 (HERSICZKY & KIRÁLY, 1972). In both cases the Ca content of the upper phase was reduced by 74% (the slight differences in the initial ion concentrations are due to the ions originally present in industrial waste waters).

It was found further that the inorganic salts, less effective in neutral medium, could be made more effective by combining them. In models simulating waste water of the meat industry the best result, an average removal of 66 ± 2.11%, was achieved with the combination of $MgCl_2$ and $CaCl_2$. An informative trial was carried out in the same model with the combination of 3 inorganic salts and an improvement of 10% was achieved in comparison to the combination of 2 salts.

Finally, cellulose activated with inorganic salts and lignites treated with sulfuric acid were also effective in reducing the COD of waste waters of the meat industry, by 50 ± 31% and ±19.5%, respectively. Further investigations are required to throw light on details of activation and the possibilities of sludge utilization.

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RAPID METHODS FOR THE DETERMINATION OF FATS IN BAKERY PRODUCTS

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To determine the fat content in bakery products, methods were sought for having low requirements for time, material and equipment, suited for serial tests and at the same time sufficiently exact. Two such methods were found: determination by acidobutyrometry and by refractometry.

This paper describes the development and application of an acidobutyrometric method consisting of the degradation of the material to be analysed with sulfuric acid of known concentration and subsequent volumetric measurement of the fat thus liberated, further of a refractometric method consisting of the extraction of the fat with alfa-bromonaphthalene at predetermined temperatures under known conditions and measuring the refractive index of the solution thus obtained.

Both methods were used to determine the fat content in various types of pastry products. The results were compared with one another and with the theoretical values obtained by the present standard method. The coefficients of variation of the two rapid methods were low, varying between 1.95 and 8.67%.

To determine the true fat content with the refractometric method, the application of a correction factor is advisable. A close correlation exists between the results obtained by the different methods and between them and the theoretical values.

Both rapid methods are highly suitable for routine tests and their time and reagent requirements are low.

The fat content of foods is understood to mean a mixture of triglycerides, fatty acids, phosphatides, extracted with solvents. They may be contaminated with hydrocarbons, organic acids, pigments, vitamins, *etc.* The fat content as determined in bakery products is composed of the fatty substances in the basic and added components (flour, margarine, milk, eggs, *etc.*). These constitute the complex chemical compound mentioned above. These basic fatty substances enter into interaction with other substances present, particularly with proteins, during the formation of the pastry and even more so at the high temperature of baking forming bonds of various strength. Solvent extraction permits only the determination of free fatty substances, while bound fats may be determined after previous liberation. The amount of fat extracted depends also on the kind of solvent applied.

There are several analytical methods known to be suitable for the determination of fats in products of the bakery industry. The classical methods of fat analysis (*Soxhlet, Weibull-Stoldt, Grossfeld* methods) were summed up by BEYTHIEN and DIEMAIR (1957). With the *Soxhlet* method in its well-known form, the material to be analysed is extracted after comminution and drying in a special extractor with ethyl ether free of water and peroxide. The solvent is evaporated and the residue is dried and weighed. This method measures

only the free fat content. A modified variant of the method was developed by *Weibull-Stoldt*. In this case, the extraction with ether is preceded by acid digestion and thereby the method serves to determine the total fat content. After appropriate preparation according to *Grossfeld*'s method, the sample is digested with hydrochloric acid, then extracted under reflux, with trichloroethylene. After evaporation of the solvent, the residue is weighed. In the *AOAC* method (ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS, 1960), the hydrochloric acid treatment is followed by diethyl ether-petroleum ether extraction. Repeated methanol-chloroform extraction is applied by SOUTHGATE (1971). The extract is filtered then the solvent evaporated. The residue is taken up in petroleum ether and the fat content is determined from an aliquot. Similarly intricate is the *Hungarian Standard* method consisting of several steps (HUNGARIAN STANDARD, 1971). The sample is appropriately prepared, treated with sulfuric acid containing ethyl alcohol, extracted in petroleum ether. The fat content is determined in an aliquot of the petroleum ether extract.

Lately, the indirect methods of fat determination have come into the fore. To mention only a few, the most modern of these are based on the principle of magnetic resonance (SLIGHT, 1970) or of infrared spectroscopy (FREEMAN, 1968). One of the indirect methods is the refractometric determination developed by JAKOBEY (1948). GOSUDARSTVENNIY STANDART (1968) is an adaptation of this technique to bakery products. PETZOLD and FREITAG (1972) have also applied this method to bakery products. Assays were made to determine fat based on measurement of density (WAHID *et al.*, 1973).

The acidobutyrometric method is entirely different from the above. It is used as a routine test in the meat and milk industries and was first applied to bakery products by KAZANSKAYA and co-workers (1975).

Because of their high time and reagent requirement, the classical methods of fat determination are not reviewed. Although, according to data in the literature, the indirect methods are accurate and rapid, the majority are too expensive to be taken into consideration.

Two methods seemed to satisfy our original aim: fat determination by acidobutyrometry and by refractometry. Hereunder the investigation carried out by these two methods are accounted for.

1. Materials and methods

1.1. Materials

The test materials were baked in the laboratory in accordance with the material consumption standard as applied in the industry. The total amount of the samples thus prepared was used in the investigation in order to avoid error due to sample taking (Table 1).

Table 1

Composition of the bakery products analysed

Materials used	Dimension	Kinds of bakery products						
		1	1a	2	3	3a	4	5
Flour BL 55	g	100	100	100	100	100	100	100
Yeast	g	2.5	2.5	3	5	5	5	5
Salt	g	1.2	1.2	1.2	1	1	0.8	0.8
Sugar	g	2.0	2.0	4.0	10	20	10	8
Margarine	g	1.0	2.0	4.0	10	15	35	55
Milk	cm^3	30	30	30	30	50	20	30
Egg	g	–	–	–	9	15.8	4.5	4.5

1 Made with milk
1a Increased fat content, made with milk
2 Enriched
3 Enriched with eggs
3a "Gugelhopf" with eggs
4 Short pastry
5 Flaky pastry

The theoretical fat content is equal to the sum of the fat contents of the basic components and additives, expressed as percentage of the dry matter.

1.2. Determination of the fat content by refractometry

In this method, the fat content of the sample to be analysed is extracted with a solvent of high refractive index, not reacting with the fat components but having a high dissolving capacity. The change in the refractive index of the extract is used to calculate the fat content by means of the following equation:

$$F = 100 \frac{v \cdot d}{g} \cdot \frac{n_2 - n}{n - n_1} \cdot \frac{100}{100 - W}$$

where

F = fat content of the sample as percentage of the solids content (%)
g = amount of sample weighed in (g)
n = refractive index of the extract
n_1 = refractive index of the pure fat
n_2 = refractive index of the solvent
v = volume of the solvent (cm^3)
d = density of the fat (g cm^{-3})
W = moisture content of the sample (%)

Using the methods as described in the literature, the differences between the values calculated and measured were considerable.

By varying the experimental conditions (temperature, volume of solvent, extraction time), finally a method was developed which gave results closely approximating the theoretical fat content. The solvent used in the experiments was alfa-bromonaphthalene.

Reagents used were:

alfa-bromonaphthalene (anal. grade)
Na_2CO_3 (anal. grade)
acetone, for cleaning the refractometer (anal. grade)

Procedure: After removal of the crust, the sample is comminuted and dried, then ground in a coffeemill. Of the sample thus prepared, 1 g (to $1 \cdot 10^{-2}$ g accuracy) is weighed into an unglazed mortar, 1 g of Na_2CO_3 and 4 cm^3 solvent at 30 °C are added. After intense rubbing for 3 min, the sample is placed in a drying oven of 100 °C. After 10 min the sample is rubbed again and filtered. The first few drops are discarded and the refractive index of the filtrate is determined in a refractometer tempered at 30 °C. The refractive index is read when no displacement of the scale is observed within one min. The refractive index of the pure solvent is measured at the same time. The refractive index of the extracted fat – if consisting of the mixture of various fats – has also to be measured. To establish the refractive index of the mixture, about 5–6 g of the sample are shaken for 15 min with the solvent (chloroform or carbon tetrachloride), three times the amount of the sample. The extract is filtered, the solvent completely evaporated, the residue dried and the refractive index of the pure fat measured. It is sufficient to carry out this determination once for every type of pastry. Naturally, in the case of a pastry of unknown composition, the above determination has to be performed. The refractive indices of the pure solvent, and of the pure fat are both measured at 30 °C. An *Abbe* refractometer was used in the experiments.

Simultaneously with the determination of fat the moisture content of the sample was also established by drying for an hour at 130 °C.

1.3. Fat determination by acidobutyrometry

With the acidobutyrometric fat determination method, the protein, carbohydrate and other components of the sample are solubilized in sulfuric acid of known concentration in the presence of iso-amyl alcohol while the fat content is liberated. When heated, the reducing effect of surface tension of iso-amyl alcohol, promotes the formation of a uniform fat layer.

The fat content is calculated by the following formula:

$$F = \frac{h \cdot 0.01133}{g} \cdot \frac{100}{100 - W} \cdot 100$$

where

F = fat content of the sample related to the solids content (%)
h = height of the fat column (scale divisions)
g = amount of weighed-in sample (g)
W = moisture content of the sample (%)
0.01133 = amount of fat corresponding to one scale division (g)

Reagents required:

60% H_2SO_4 (density = 1.5 g cm^{-3})
iso-amyl alcohol (anal. grade)

The sample is prepared as for the refractometric method. Two g of the prepared sample are weighed (to $1 \cdot 10^{-2}$ g accurancy) into an Erlenmeyer flask of 25 cm^3, 10 cm^3 of the 60% sulfuric acid are added and stirred. The flask is placed in a water bath of 80 °C, for 20 min. The mixture is stirred from time to time with a glass rod, then transferred quantitatively into a Gerber butyrometer and the Erlenmeyer flask is rinsed with 9 cm^3 sulfuric acid. One cm^3 of iso-amyl alcohol is added and, if necessary water enough to bring the fat layer into the graded part of the butyrometer. The butyrometer is then stoppered with a rubber stopper and thoroughly shaken for 3 min, then placed, in an inverted position, for 5 min into the water bath of 80 °C. After centrifuging for 5 min, it is placed again into the water bath of 80 °C and the height of the fat column is read in the graduated part while still warm.

1.4. Determination of fat according to the Hungarian Standard

In the method described in the HUNGARIAN STANDARD (1971), the preparation of the sample and the determination of its moisture content is carried out in the same way as with the refractometric method. Ten g of the sample, weighed, to $1 \cdot 10^{-2}$ g accuracy, into a flask used for the determination of the iodine number. Thirty ml of sulfuric acid, containing ethyl alcohol (100 volume units of 96% ethyl alcohol are mixed with 5 volumes of concentrated sulfuric acid, freshly made), are added. The flask is placed into a drying oven of 105 °C for 45 min and shaken every 10 min. After digestion, 10 cm^3 of 96% alcohol are added and 50 cm^3 of petroleum ether, after cooling. The mixture is then thoroughly shaken, allowed to stand for 2 h while shaking every half hour. To separate completely the petroleum ether layer, the flask is filled up nearly to its neck with tap water, shaken and allowed to stand over night. In a 25 cm^3 beaker previously dried in an oven of 105 °C and weighed to $1 \cdot 10^{-4}$ g accuracy 20 ml of the pure petroleum ether layer are pipetted. The petroleum ether is evaporated at a temperature not higher than 40 °C. It is then placed into a drying oven of 105 °C for 10 min in order to remove the last trace of petroleum ether. The beaker is then cooled in a desiccator and weighed to $1 \cdot 10^{-4}$ g accuracy.

Table 2

Evaluation of the fat content of different bakery

Method	Type					
	Milky			Enriched		
	$\bar{x}$	s	d	$\bar{x}$	s	d
Standard	2.84	0.15	0.56	5.36	0.23	0.62
Refractometric	2.19	0.19	0.46	4.84	0.15	0.45
Acidobutyrometric	2.58	0.14	0.32	5.13	0.16	0.31

$\bar{x}$ = average of 20 individual measurements; s = standard deviation;

Reagents required:

96% ethyl alcohol (anal. grade)
conc. H_2SO_4 (anal. grade)
petroleum ether (boiling point below 80 °C).

The fat content of the sample analysed is calculated by the following equation and is expressed as per cent related to the solids content:

$$F = \frac{(A - l) \cdot f}{a - \frac{f}{d}} \cdot \frac{100}{g} \cdot \frac{100}{100 - W}$$

where

A = volume of petroleum ether added to the sample (cm^3)
l = loss of petroleum ether during retention time (cm^3)
a = volume of the petroleum ether extract pipetted for evaporation (cm^3)
f = amount of fat recovered after evaporation (g)
d = density of the fat (assumed to be 0.9)
g = amount of sample weighed-in (g)
W = moisture content of the sample (%)

2. Results

The methods of analysis were applied to various products of the baking industry. Short and flaky pastry enriched with milk or eggs were prepared in the laboratory and analysed using the HUNGARIAN STANDARD (1971), the acidobutyrometric and the refractometric methods. Each kind of product was analysed in 20 parallels by each of the three methods. The evaluation of

products determined by different methods

of pastry								
Enriched with eggs			Short			Flaky		
$\bar{x}$	s	d	$\bar{x}$	s	d	$\bar{x}$	s	d
9.98	0.38	1.10	23.11	0.67	2.03	30.81	0.57	1.91
9.41	0.32	0.90	23.17	0.37	0.96	30.45	0.40	0.99
9.85	0.30	1.00	23.13	0.47	1.22	30.14	0.72	1.83

$d = x_{max} - x_{min}$-difference in the maximum and minimum of 20 individual measurements. Calculated fat content (%): milky 2.69; enriched 5.08; enriched with eggs 9.63; short 23.63; flaky 37.90.

results is contained in Table 1, while the significance of differences between average values is shown in Table 2.

Standard deviations of the three methods were established on the basis of analysis carried out in several laboratories. Bakery products of the same

Table 3

Significance of difference between average fat contents

	Milky		Enriched		Enriched with eggs		Short		Flaky	
	R	A	R	A	R	A	R	A	R	A
S	***	***	***	***	***	Ø	Ø	Ø	*	**
R	–	***	–	***	–	***	–	Ø	–	Ø

*: $P \geq 95$ (significant)
**: $P \geq 99\%$ (highly significant)
***: $P \geq 99.9\%$ (very highly significant)
Ø: $P < 95\%$ (not significant)

R: refractometric method
A: acidobutyrometric method
S: standard method

Table 4

Results obtained by acidobutyrometry in the collaborative tests

Type of product	Results obtained in different laboratories (%)					Calculated fat content (%)	Average of measured values ($\bar{x}$)	Standard deviation (s)
	A	B	C	D	E			
Milky	3.02	2.53	3.10	2.80	2.59	2.69	2.81	0.313
Enriched	5.08	4.88	5.60	5.08	4.94	5.08	5.06	0.411
Enriched with eggs	10.27	9.42	9.97	9.79	9.81	9.63	9.88	0.331
Short pastry	24.04	23.09	22.77	22.29	21.36	23.63	22.68	1.325
Flaky pastry	30.25	29.14	27.53	31.08	29.17	37.90	29.64	1.218

The values obtained in different laboratories are the averages of 5 parallel measurements

kind were prepared for analysis (crust removed, comminuted, dried, finely ground) and the crumbs sent to several laboratories specialising in food analysis. Tables 4 and 5 give the results of these tests and their evaluation.

The results include those obtained in the laboratory of the author as well. The results are averages of five parallel measurements.

The results of the test series were used in determining the correlation between calculated values and the averages obtained by the different methods.

Table 5

Results obtained by refractometry in the collaborative tests

Type of pastry	Results obtained in different laboratories (%)			Calculated fat content (%)	Average of measured values ($\bar{x}$)	Standard deviation (s)
	A	B	C			
Milky	2.44	2.68	2.28	2.69	2.43	0.226
Enriched	4.78	4.03	3.85	5.08	4.25	0.472
Enriched with eggs	9.30	9.76	–	9.63	9.47	0.303
Short pastry	22.95	22.46	21.91	23.63	22.44	0.497
Flaky pastry	28.73	27.86	27.39	37.90	28.01	0.632

The values obtained in different laboratories are the averages of 5 parallel measurements

Table 6

Fat contents of different types of bakery products as obtained by different methods

Calculated fat content (%)	Measured fat content (%)		
	Standard method	Refractometry	Acidobutyrometry
2.69	2.84	2.19	2.58*
2.69		2.43	2.81
3.55		2.81	3.13*
5.08	5.36	4.84	5.13*
5.08		4.25	5.06
9.63	9.98	9.41	9.85*
9.63		9.47	9.88
12.32		11.83	12.60*
23.63	23.11	23.17	23.13*
23.63		22.44	22.68
37.90	30.81	30.45	30.14*
37.90		28.01	29.64

Results marked * are averages of 20 parallel measurements. Of the other results those obtained by refractometry are averages of 15, those obtained by acidobutyrometry of 25 measurements

Table 7

Correlation analysis of the results obtained by three different methods

Independent variable (x)	Dependent variable (y)	Number of data pairs (n)	Coefficient of determination (r^2)	Equation of the regression line ($y = a + bx$)
Calculated value	Standard method	4	0.9995	$y = 0.445 + 0.963x$
Calculated value	Refractometry	10	0.9985	$y = -0.356 + 0.984x$
Calculated value	Acidobutyrometry	10	0.9983	$y = 0.183 + 0.970x$
Standard method	Refractometry	5	0.9997	$y = -0.641 + 1.016x$
Standard method	Acidobutyrometry	5	0.9996	$y = -0.126 + 0.991x$
Refractometry	Acidobutyrometry	12	0.9979	$y = 0.464 + 0.998x$

The numbers of pairs of data are average values

Table 8

Results of on-line acidobutyrometric measurements

Type of pastry	Calculated fat content (%)	Measured fat content (%)	Standard deviation (s)
Milky	2.69	2.56	0.18
Enriched	5.08	5.31	0.32
Enriched with eggs	9.63	9.75	0.88
Short pastry	23.63	22.52	0.60
Flaky pastry	37.90	29.45	0.91

The measured values are averages of 10 parallel determinations

In correlation analysis, data on brioche and milk-loaf were also taken into account. The average values used in the calculations are shown in Table 6 and the correlations between the different methods are summed up in Table 7.

Testing the significance of the differences between the slopes of the regression lines has shown that the deviation of the slopes from the average is not significant at the 95% probability level.

The methods described were applied in the analysis of the dough prior to baking in on-line control. The refractometric method was not satisfactory for this purpose while the results obtained by acidobutyrometry were encouraging (Table 8).

In dough analysis, the solids content needed to calculate the fat content was determined in a *Glutork*-type apparatus in a 5-g sample dried for 4 min (HALMOS-MAXIMOVA, 1979).

3. Conclusion

3.1. Evaluation of the refractometric method

The refractometric fat determination method improved by the author is simple, rapid and sufficiently accurate. The results obtained by this method are somewhat lower than those of other methods. This may be explained by the fact that, while with both the acidobutyrometric and the standard method, fat determination is preceded by acid digestion, with the refractometric method, the procedure is reduced to solvent extraction and measurement of the refractive index. To prove this assumption experiments were carried out in which refractometric determination was preceded by acid digestion. The results obtained corresponded to the theoretical fat content, but the procedure was extremely lengthy. If the results obtained by the rapid technique are corrected by a constant (+0.36) calculated from the regression line of the method, the true fat content is obtained.

Exact setting of the temperature of the refractometer is decisive in the correct determination of the fat content. A difference in the temperature of 0.1% brings about a difference of 0.2% in the fat content. Important is also the accurate dosage and appropriate temperature of the solvent. A difference of 1 °C causes a difference of $5 \cdot 10^{-4}$–$6 \cdot 10^{-4}$ in the refractive index of the solvent. Since the solvent is in contact with the sample only for a short time, to achieve complete extraction of the fat, the sample has to be vigorously rubbed. Extraction is promoted by the application of added Na_2CO_3 and an unglazed mortar. The difference between the results obtained by the use of glazed and unglazed mortars, resp., manifests itself in a difference of $4 \cdot 10^{-4}$ in the refractive index. In addition to the strict adherence to the instructions the accuracy of the method depends on the systematic error of the refractometer used (in our case the systematic error inherent in the *Abbe* refractometer used was $2 \cdot 10^{-4}$).

3.2. Evaluation of the acidobutyrometric method

According to the pertinent literature as well as to the personal experience of the author, the acidobutyrometric method is rapid, inexpensive, sufficiently exact and extremely suitable for routine tests. In addition to the analysis of the final product, the procedure is also suited for on-line control of the dough.

The concentration of the sulfuric acid is an important factor of the precision of the method. A 50% sulfuric acid solution is too diluted to dissolve completely the proteins present, therefore, the result obtained with it are lower than the true value. A solution of 70% carbonifies sugar-like substances, these become dispersed in the fat and may, after centrifuging, form a plug between the sulfuric acid layer and the fat column. If a plug is formed, the analysis has to be repeated.

If the instructions are exactly observed the accuracy of the method is limited by the readability of the butyrometer. Half a division is safely readable by the naked eye.

3.3. Correlation between the methods

The average results obtained by the two rapid methods described above give a good approximation of the theoretical values. In the case of the refractometric method, however, the result has to be corrected by a factor.

The calculated standard deviations in both the acidobutyrometric and the refractometric methods are generally lower than those with the standard method. Exemptions are the standard deviations calculated from the results obtained by the acidobutyrometric method for pastry of high butter content and by the refractometric method for milk-containing products. It is surprising that the deviations calculated from the results obtained by acidobutyrometry in different laboratories for milk- and egg-containing, as well as enriched bakery products were hardly higher than those obtained within a laboratory, while the deviations increased substantially with short and flaky pastries.

The standard deviation as calculated from the results of refractometric measurement carried out in different laboratories increased somewhat over the deviations obtained from the results of one laboratory.

A very good correlation was found between the results obtained by the two methods tested, those of the standard method and the theoretical values of the fat content. The calculated regression lines are not applicable to products made of flaky pastry because the loss of fat upon baking in these products may amount to one fifth of the initial fat content. In paired data used in correlation analysis in relation to the theoretical values, the results obtained with flaky pastry were not taken into consideration because they would have strongly distorted the correlations between other products.

The control tests of the two rapid methods have shown these two methods to be suitable for fat determination in bakery products. Their advantage lies in their low time requirement. Fifteen routine analysis may be carried out within one work-shift by both methods. The standard method, since it includes an over-night standing period, permits of 30 analyses within 2 days and it takes a longer time to get the information.

The expense of reagents for the two rapid methods is low. If the costs of reagents for one analysis by the standard method are considered as 100%, those for the acidobutyrometric method amount to 0.37%, while those for the refractometric method to 16.4%. The acidobutyrometric method proved to be suitable for on-line control and provided information in a very short time thus this method was suggested for on-line control as well as for use as a standard method.

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HIGH-RATE SEDIMENTATION IN THE FOOD INDUSTRY

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One of the methods used in the food industry to separate inhomogeneous disperse systems is sedimentation. Owing to long detention times, the traditional settling devices are not always suited to the separation of the biochemically unstable semi-products of the food industry. The same applies to equipment based on the principle of centrifugation in relation to flocculent and gelatinous substances. In order to reduce these setbacks a high-rate type of settling equipment, called "*Sedikon*", was developed and tested under laboratory, pilot plant and industrial conditions.

The lamella separator type equipment was found to reduce significantly ($P \geq 99.9\%$) the initial suspended solids concentration in raw sugar refinery effluent, washing water of pig slaughterhouses, mechanical treatment of starch effluents, in the washing waters of potatoes, tomatoes and sugar beets. A poorer result was obtained only with concentrates of fresh must and with thin dregs containing yeasts. These results were achieved with 4–6 h retention times.

The trend of steadily increasing the nutritional value and taste of foods necessitates the constant improvement of processing operations.

Wet processing prevails in the food industry and is aimed at the possibly perfect separation of inhomogeneous fluid systems. The simplest and at the same time the most ancient technique of wet separation is sedimentation.

In the course of technical development several types of machines have been developed, that are capable of producing accelerations several times the gravitational value by means of the centrifugal force. Besides increasing unduly the manufacturing costs, these devices are in a number of cases unsuited for processing certain substances, such as the washing waters of most agricultural products and the semi-products of the food industry containing flocculent or gel-like materials. In the first case the valuable machinery is damaged by wear. In the second case comminution by friction interferes with further processing. *E.g.* sugar beet flume water containing flocs, once treated in hydrocyclone, cannot be separated further by sedimentation (Hersiczky & Király, 1972). To overcome these difficulties experiments were carried out to develop equipment in which suspended solids precipitate without any mechanical effect and settle together with the particles causing wear.

1. Equipment and methods used in the experiments

1.1. Equipment

The settling tanks and basins used in the food- and related industries vary widely in design and may be square, rectangular, or circular in plan. The direction of flow through them may be horizontal, radial and vertical

(BARTHA *et al.*, 1976). These familiar types are unsuited for use in the food industry mainly because of their poor efficiency and low capacity. Long detention times may result in fermentation or anaerobic decomposition impairing quality.

The fall velocity determining capacity is described – regardless of equipment type or medium – by the *Stokes* Law (HOLLÓ & SZEJTLI, 1958; TÖRÖK, 1976):

$$V = \frac{H}{t} = \frac{2}{9} g \frac{d^2}{4} \frac{\varrho_1 - \varrho_2}{\eta}$$

where

V = fall velocity, cm sec^{-1}
H = height of fall, cm
t = settling time, s
ϱ = density of the dispersion medium and of the disperse phase, g cm^{-3}
d = particle diameter, cm
η = dynamic viscosity of the medium, g cm^{-1} s^{-1}
g = acceleration by gravity, g cm s^{-2}

The settling time t will be seen to depend, besides the fall velocity, on the height of fall, and thus the sedimentation chamber was divided by baffles. To find the most advantageous arrangement of the baffles, prisms of 45 cm height and 10 cm length, but of different width were made. The prisms of 3.5, 5.0 and 7.0 cm width were inclined from 90° and 30° to the vertical. Settling in the various prisms was investigated with sugar beet flume water containing sand and floccular precipitate, and treated with lime. The results obtained with the vertical and most advantageously inclined prisms are illustrated in Figs. 1 and 2.

A 300 l random sample of sugar beet flume water was used for the experiments, in order to eliminate the distortion caused by fluctuations in the concentration and composition of the water. The flume water of 28.4 $\pm$ 0.21 g cm^{-3} density was adjusted with $Ca(OH)_2$ to pH = 11 and the solids and flocs were kept in suspension by slow agitation. The layer thickness of the liquor clarified under the above conditions was determined by five parallel measurements for each prism and the results showed little variation about the mean. The highest coefficient of variation was $\pm 1.14\%$.

In Fig. 1, after a settling time of 20 min the prisms of 3.5, 5.0 and 10 cm width show no dimensional effects. A significant difference at the probability level of 99.9% was found only with the prism of 7 cm width. This dimension had an adverse effect on the fall velocity studied. On the other hand, the results in Fig. 2 show a significant difference for the prism of 10 cm width. The inclination of the prism by 45° produced a sludge flow pattern resulting

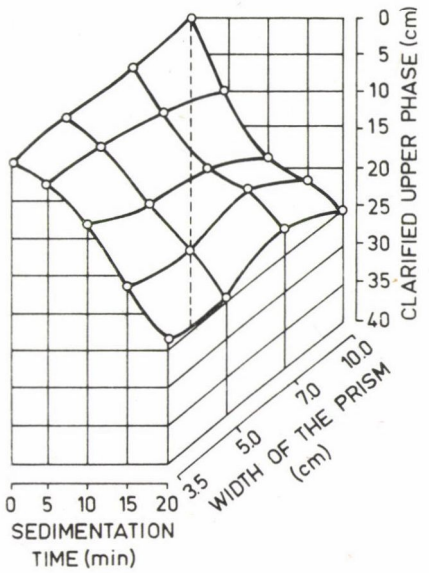

Fig. 1. Increase of the clarified upper phase of sugar beet flume water depending on the sedimentation time and width of the vertical plexiglass chamber used in the experiment. Constants of the chamber 10×45 cm

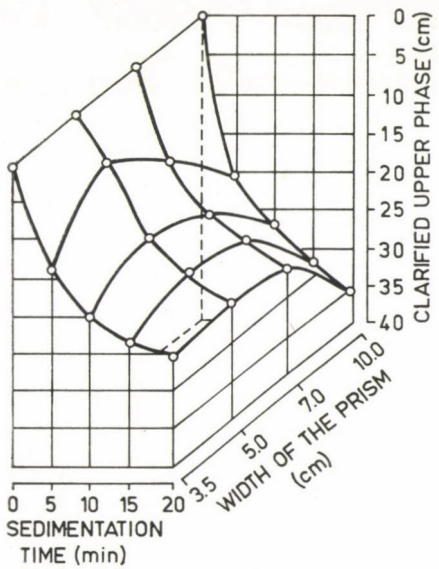

Fig. 2. Increase of the clarified upper phase of sugar beet flume water depending on sedimentation time and width of the inclined plexiglass chamber used in the experiment. Constants of the chamber 10×45 cm

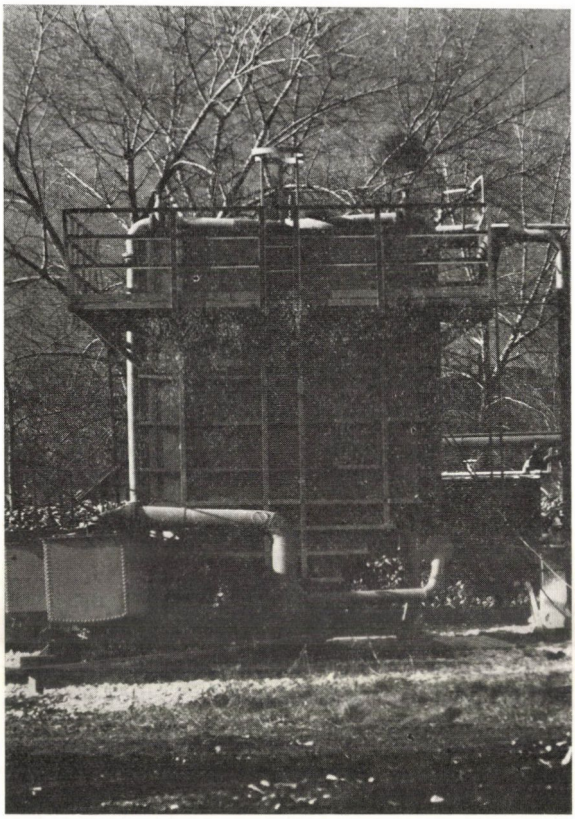

Fig. 3

in highest fall velocity and sludge concentration. The same spacing was thus adopted for the plant-scale prototype (Fig. 3), with the lamellae inclined at 45°.

The effluent is introduced on two sides of the sedimentation unit, while the sludge is drawn off at the base of the centre line, the clarified liquor at the top end. The conditions prevailing in this system permit any kind of floccular precipitate to separate and settle to the bottom in the sedimentation chamber divided by baffles. Outflow at the top end of the centre line enables the removal of particles, which owing to their size and specific gravity do not settle by gravity. This is effected by the sediment descending from the baffles and encountering in the central segment of the chamber the non-settling particles carried by the upward flow. This process, which may be called sludge rectification, ensures a clear phase not achievable either by the application of a scraper or of settling tanks.

A scale difference of 50 exists between the laboratory-, pilot- and industrial size equipment. Thus, the *Sedikon* type sedimentation units are of

20, 1000 and 50 000 litres volume (HERSICZKY *et al.*, 1970). The spacing of the PVC lamellae is, in the same order, 1.5, 3.5 and 10 cm.

1.2. Methods

1.2.1. Determination of the suspended substance with filter paper. Preweighed Macherey-Nagel folded filter paper, No 615/1/4, was used in the experiments. Exactly 100 ml liquor was filtered. The filter paper was dried at 105 °C to constant weight and weighed to two decimals. The difference in the weight as measured and the initial weight of the filter paper, multiplied by ten gave the suspended material in g l^{-1} units.

1.2.2. Determination of the total suspended material with angle-head centrifuge. An angle-head centrifuge with tubes of 50 ml volume was used. After centrifuging for 10 min at 5 000 rpm the tubes were decanted and weighed. Knowing the weight of the tube and the amount of liquor weighed in, the wet sediment could be calculated. (This wet weight is called in the wine industry *sediment* and is expressed in g l^{-1} units.) By determining the solids content of the sediment the quantity of the suspended solids can be calculated.

1.2.3. Determination of the wet volume of the suspended material. The effect of high-rate sedimentation was checked also by the change in the volume of the suspended material settling during 30 min in a graduated cylinder. The advantage of this simple test is that no laboratory is required and the information is available immediately. The sample need not be transferred and stored, thus, biochemical changes need not be accounted for.

1.2.4. Measurement of the discharge. The in- and outflow discharge was found by measuring the time needed to fill the unit of given volume at different valve positions. (Owing to the presence of suspended particles, the flow meters available are difficult to use.)

1.2.5. Measurement of the mass flux. Changes in the mass of suspended material in unit time can be evaluated by multiplying the discharge and the suspended material concentration, in accordance with the following dimensional equation:

$$\frac{1}{\text{min}} \cdot \frac{g}{1} = \frac{g}{\text{min}}$$

1.2.6. pH measurement. The pH of the inhomogeneous systems was determined with the universal *Lachema* indicator paper and with *Stupan* indicator sensitive in alkaline medium.

1.2.7. Determination of the dissolved material. The dissolved organic and inorganic content of the suspensions to be analysed was determined with a *Zeiss* refractometer used in the sugar and vegetable oil industry and expressed as per cent by weight.

2. Results

2.1. Laboratory-scale experiments

2.1.1. Reduction of suspended substances in sugar refinery effluents. The suspended solids in sugar refinery raw liquors make further processing, particularly filtration and thickening difficult. The introduction of continuous diffusion increased the suspended material in the liquors (MALATINSZKY, 1975). This is caused in the continuous diffusers, independent of their type, by the diffusing liquor flowing in counter-current to the slowly moving cosettes which are comminuted by friction. The amount of shreds increases with the advance of the season, particularly if frost sets in. The possibility of reducing the suspended material was tested with the model equipment of 20 l during 12 weeks on two occasions per week. The results related to the raw liquor introduced and the sediment obtained are shown in Fig. 4.

As may be seen in the figure, the great change in the suspended material content of the liquor occurs during the 8th week. Therefore it seems advisable to carry out the analyses separately for the two periods. However, concerning the results of high-rate sedimentation it was satisfying to observe that the resulting upper phase was free of sediment in both periods.

This output was achieved at 5 l h^{-1} discharge, or at 4 h retention time. In the first experimental period the sedimented cosette shreds were of 292.0 $\pm$ $\pm$ 76.7 cm^{-3} l^{-1} density from the raw liquor containing 35.8 $\pm$ 18 cm^{-3} l^{-1} wet sediment. In the second period the density of the liquor was of 124.4 $\pm$ 42.7 and that of the sediment 838.6 $\pm$ 117 cm^{-3} l^{-1}. The differences in the mean values exceeded in both cases the 99.9% probability level.

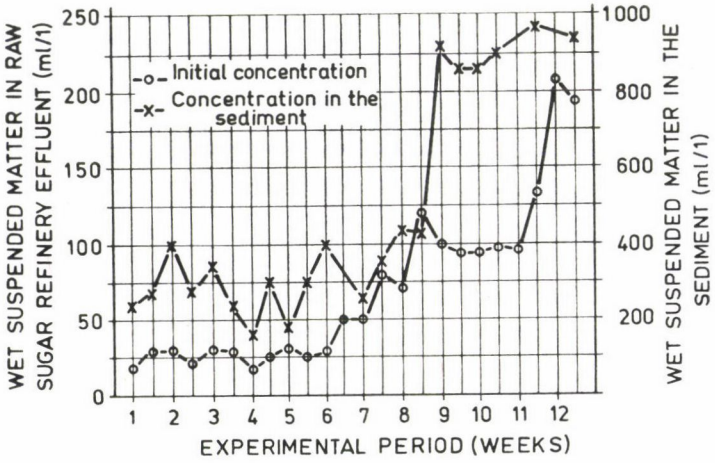

Fig. 4. Wet suspended matter in the raw sugar refinery effluent and its change in the course of high-rate sedimentation

2.1.2. Removal of the solid particles from the pig washing water in the slaughterhouse. Since the pig washing water may contain faecal matter it is important to remove the solid particles as soon as possible, and with them any pathogenic microorganisms present. The experiments were continued for 6 months. The washing water contained originally 5.4 ± 0.6 g l^{-1} solids, which was reduced at a discharge of 5 l h^{-1}, or 4 h retention time to 3.2 ± 0.12 g l^{-1}. Flocculating agents were not used. The density of the sediment amounted to 8.9 ± 0.92 g l^{-}. From the entering solids flux of 27 g l^{-1}, 12.8 g remained in the upper phase. The viable cell count of the effluent was 10^6 cm^{-3}. By the t test the difference in the upper and lower phases was found to be significant at the 99.9% probability level.

2.2. Pilot plant experiments

2.2.1. Reduction of the sediment content of must and dregs. Grape cultivating and processing plants use high pressure and continuous presses in growing numbers because of manpower shortage and in order to increase the must output. High pressure, however, causes an increase in the fibre content of the musts in comparison with the traditional presses. Suspended material of this character impairs the quality of the wine and increases the amount of dregs. To separate this matter fractionated sedimentation is applied in concrete tanks

Table 1

Concentration and efficiency relations in the pilot size SEDIKON *equipment at different input rates in must dregs*

Flow rate, l h^{-1}	Sediment in the			Efficiency, %
	Input	Upper phase	Concentrate	
	g l^{-1}			
60	102.5	17.5	125.1	92.9
60	162.5	8.0	173.0	95.0
60	162.5	8.9	193.0	94.6
120	89.2	19.7	219.0	78.0
160	30.4	9.2	73.2	69.0
180	105.2	20.4	118.0	88.0
180	109.6	13.0	138.0	88.1
180	136.9	20.8	149.0	84.8
180	132.0	24.1	158.1	81.7
420	96.4	41.0	175.0	55.4
Mean	112.7	18.3	152.1	
Standard deviation	±37.8	±9.3	±39.3	

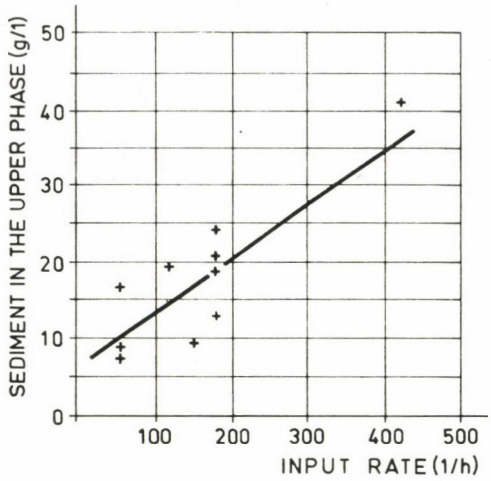

Fig. 5. Regression correlation between the input rate and the suspended matter content of the upper phase in must dregs experiments

prior to fermentation. The aim of the experiments was to establish whether the sediment obtained during 24 h fractionated sedimentation may be concentrated with the *Sedikon* equipment, and whether it is possible to reduce the retention time in fresh must and thin dregs sedimentation. Another motive of the experiments was the aim of developing continuous operation (MERCZ, 1975).

2.2.1.1. Further concentration of sweet must sediments. – Concentration and efficiency relations in the must sediment during further high-rate sedimentation are given in Table 1.

The concentration of both phases changed over the initial concentration. However, as established by the t test, the differences were significant only between probability levels 95 and 98%.

The other characteristic of results is efficiency, which is the relation of sediment in the lower phase to the total suspended material introduced during unit time, or in other words the ratio of mass flux rates. Such results are possible only by the "sludge rectification" as mentioned before, preventing the rise of the non-settling gel substance and thereby reducing the sediment content of the upper phase. This effect is, however, decreased if the flow rate in the central segment is increased. The experiments were carried out at different flow rates. The sediment values in the upper phase were graphically represented (Fig. 5).

The regression equation of the set of data is

$$Y = 6.5 + 0.07\,X$$

This means that increasing the discharge by 7 $l\,h^{-1}$ decreases the sediment content of the upper phase by 1 g l^{-1}. The correlation coefficient of the relation is 0.8.

2.2.1.2. High-rate sedimentation of fresh must. – The fresh must obtained by pressing was settled at two input rates in the pilot size equipment, drawing

Table 2

Sediment in fresh musts treated in the pilot size SEDIKON *equipment*

Flow rate, $l\,h^{-1}$	Sediment in the			Efficiency, %	Number of experiments
	Input	Upper phase	Concentrate		
	g l^{-1}				
180	38.0	4.2	57.2	89.0	1
180	117.0 ± 15	19.5 ± 5	166.2 ± 18	83.5	4
610	107.0 ± 28	56.2 ± 16	113.0 ± 31	47.6	9
610	104.7 ± 30	43.4 ± 19	121.4 ± 33	58.3	7

on the experiences described in the foregoing chapter. The experiments were evaluated every week. The averages of the parallel experiments, their standard deviations and the efficiency values are shown in Table 2.

In the successive rows of the table the result of the parallel experimental runs No. 1, No. 4, No. 9 and No. 7 are given, resp.

The operation of the traditional grape presses is approximated most closely by piston pumps. The results shown in the first row were obtained in the juice thus gained. For the further experiments, where the input rate was 180 and 610 $l\,h^{-1}$, the juice was obtained on a screw press. It was found by the t test that the difference between the data given in the second row and the original juice is significant at 95–99% level for the lower phase and at 99.9% for the upper phase. At increased input rates the efficiency decreased similarly. Efficiency became so poor that only the upper phase differed significantly from the input juice ($P \geq 99.0\%$).

2.2.1.3. Repeated sedimentation of dregs. – The next step in grape processing is the treatment of the dregs in the fermentation tanks. Since the suspension called thin dregs contains an important amount of wine, its further processing is a question of economics. For reclaiming the wine filter presses are generally used the output of which is, however, very low because of the presence of flocculated and gelatinous matter. Consequently, this operation suffers unduly long delays during which undesirable biochemical processes occur.

The aim of the experiments was to prepare from the viscous material a diluted suspension, in order to increase the output of the filter presses. The results of these experiments are summarized in Table 3.

Table 3

Fractionation of wine dregs by high-rate sedimentation

Flow rate, l h^{-1}	Sediment in the			Efficiency, %	Number of experiments
	Input	Upper phase	Concentrate		
	g l^{-1}				
400	162.5 ± 64.9	6.1 ± 2.8	318.3 ± 72.1	96.0	6
450	266.7 ± 79.0	25.0 ± 14.3	469.0 ± 98.2	89.0	4
600	306.1 ± 87.0	79.8 ± 32.0	548.7 ± 99.6	86.9	5

Analysis of the results by the *t* test has shown the upper phases to differ from the input material significantly at 99% probability. The lower phases differed at a lower level of probability. The differences in the order of increasing input rate were significant at 98–99, 95–98 and 98–99%, resp.

2.2.2. Mechanical treatment of starch industry effluents. Starch syrup is a mixture of a variety of carbohydrates, mainly of dextrin, maltose and d-glucose. Because of the uneven heat- and material transfer in the converter, some non-degraded starch may also remain in the hydrolysate. It is removed during filtration with the filter aids. The filter cake goes into the waste with the washing waters and here some of the carbohydrate components dissolve.

The aim of the experiments was to remove some of the heavier inorganic and organic components in order to promote re-use of the aqueous upper phase in the production of starch suspension. The experiments were carried out on two occasions for one months each. The results are given in Table 4.

The difference for both the upper and the lower phase was significant at the 99.9% probability level compared with the input. The suspended matter in the upper phase was floating starch gel. In addition to the 0.65 ± 0.27 and 0.31 ± 0.19% suspended carbohydrates, further 1.2 ± 0.33 and 0.9 ± 0.25% of organic matter was found by refractometer. The re-use of the latter reduces the starch consumption for unit product.

Table 4

Average results of 25 mechanical treatment experiments with starch industry effluents

Flow rate, l h^{-1}	Suspended solids in the			Efficiency, %
	Input	Upper phase	Concentrate	
	g l^{-1}			
180	21.8 ± 9.6	6.5 ± 2.7	83.0 ± 13.0	76
180	15.8 ± 7.8	3.1 ± 1.9	67.0 ± 9.5	84

Table 5

Results of flume and washing water sedimentation in potato and tomato processing (at pH 6.5 and 11.0, resp.)

Flow rate, $l\ h^{-1}$	Suspended solids in the			Efficiency, %	Number of experiments
	Input	Upper phase	Concentrate		
	$g\ l^{-1}$				
in potato processing					
2000	1.33 ± 26	6.23 ± 2.8	625.0 ± 51	96	6
in tomato processing					
5000	1.88 ± 0.36	0.31 ± 0.11	9.83 ± 0.65	86.6	18

2.2.3. Reduction of the suspended material in potato and tomato flume and washing waters. Agricultural products are generally washed prior to processing. In the case of potatoes, tomatoes and sugar beet the water serves the purpose of transport as well. Experiments were carried out to treat the waste waters of potato and tomato processing. In the case of potatoes the washing water was used in its original form, the tomato washing water was alkalified with milk of lime. The results are given in Table 5.

Owing to the larger particle size of the suspended material, the difference between the input water and the product was significant at the 99.9% level.

2.3. Operation of the industrial-scale equipment

2.3.1. Fractionation of must and dregs. The equipment of 50 000 l volume, inserted between the press and the fermentation plant, operated for 16 days during the grape processing season. The input rate differed as a function of the quantity of grape received (500 to 9 000 $l\ h^{-1}$). Its operation was twice interrupted because of fermentation.

The equipment was checked for efficiency every other day by means of samples taken. The results of 24 samples are summarized below:

Input	$31.7 \pm 9.6\ g\ l^{-1}$ wet sediment
Upper phase	$14.5 \pm 5.4\ g\ l^{-1}$ wet sediment
Concentrate	$159.5 \pm 32.9\ g\ l^{-1}$ wet sediment

No efficiencies have been attached to the data, since the discharges could not be measured reliably under plant conditions.

In the next step the dregs formed during fermentation, and accumulating at the bottom of the tank, were fractionated. From the available dregs about 60% was thin dregs and 40% thick suspension. On the basis of 18 samples the following data were obtained:

Input	130.3 ± 67.8 g l^{-1} wet sediment
Upper phase	29.3 ± 12.0 g l^{-1} wet sediment
Concentrate	284.6 ± 49.7 g l^{-1} wet sediment.

The filtration behaviour of the upper phase was improved at the 99.9% probability level over that of the input, although the concentrations of the two suspensions differed only at the 98–99% probability levels. The average concentrate has attained, on the other hand, the 99.0% probability level.

Finally the sediment formed during the last clarification prior to bottling was fractionated. This sediment amounts to about 2–4%, therefore the total amount formed during a year is substantial (OLÁH, 1976; ÁSVÁNY, 1976). This sediment, in contrast to the thin dregs, does not contain degrading yeasts, but the acceleration of filtration is a factor in improving productivity. The averages of 40 samples are as follows:

Input	76.0 ± 18.0 g l^{-1} wet sediment
Upper phase	2.0 ± 0.14 g l^{-1} wet sediment
Concentrate	358.0 ± 65.2 g l^{-1} wet sediment.

Both fractions differ significantly from the input at the 99.9% probability level. Owing to the advantageous consolidation and settling properties of the material it was possible to separate the concentrate in 20% by volume. The filtration behaviour of the thin phase improved, too.

2.3.2. Clarification of the sugar beet flume and washing water in view of recirculation. As mentioned in the introduction already, an attempt was made to increase the treatment rate of sugar refinery effluents with hydrocyclones (CZIRFUSZ, 1962). However, because of erosion and dispersion effects these experiments had to be abandoned. For this reason plant scale trials were performed with high-rate sedimentation for this purpose. On this occasion the accurate determination of the flux rates of the fractions was also possible.

To determine the ratio of volume distribution and of concentration batch experiments were also carried out. The waste water was adjusted to pH = 11 with milk of lime and then introduced into the equipment at a rate of 4,000 l min^{-1}. After 20 min retention time following complete filling the upper phase, amounting to about 40 000 l, was drained and samples were collected. Similarly samples were taken from the lower phase of about 10,000 l. This batch experiment gave the following results:

Input	18.0 g l^{-1} dry residue
Upper phase	3.0 g l^{-1} dry residue
Concentrate	63.5 g l^{-1} dry residue

The sediment in the lower phase comprises 87.6% of the total solids content of the original waste water.

In regular operation the sedimentation equipment was used to clean the effluent from the floatation circuit, with the following result:

Input	11.9 ± 0.69 g l^{-1} solids
Upper phase	2.3 ± 0.04 g l^{-1} solids
Concentrate	158.0 ± 5.60 g l^{-1} solids.

The hourly treatment rate of the equipment was $240 \cdot 10^3$ l, of which about $200 \cdot 10^3$ l were recirculated. The residual cca $40 \cdot 10^3$ l sediment was finally removed from the plant area and disposed of in filter beds. The removal efficiency calculated by the operator, was 84%. Data recorded during a whole sugar beet processing campaign have shown an advantageous effect of sludge rectification on concentration.

3. Conclusions

The results of experiments carried out so far in various branches of the food industry show that the *Sedikon* type high-rate sedimentation equipment reduces the sedimentation time from the generally applied 24 h to 4–6 h, reducing at the same time the danger of biochemical degradation. Fermentation was observed only on two occasions with freshly pressed grape juice. In subsequent designs the inner, dividing part of the settling chamber should be made removable, permitting the lower part of the chamber, where the flow rate is slower and the danger of infection centres greater, to be thoroughly cleaned with a water jet.

A further conclusion is that besides centrifuges, equipment is needed which, instead of dispersing the suspended matter, removes the flocs and gels with high efficiency, partly by the counter flow sludge rectification effect.

As regards the fractionating effect, the difference over the entering concentration was found significant at the 99.9% probability level in the case of raw sugar refinery effluents, pig washing waters, mechanical treatment of starch factory effluents, flume and washing water of sugar beet, potatoes and tomatoes, and the dregs of wine. Poorer results were obtained only with the concentrate of fresh musts and the yeast containing thin dregs. On the whole the results hitherto obtained are encouraging and incentive.

*

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SIMULTANEOUS QUANTITATIVE DETERMINATION OF SORBIC AND PROPIONIC ACIDS BY GAS CHROMATOGRAPHY IN PRESERVATIVE-CONTAINING BAKERY PRODUCTS

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An analytical method has been developed for the simultaneous quantitative determination of calcium propionate and potassium sorbate in preservative-containing bakery products. A specially constructed steam distillation apparatus was used to extract the preservatives. The distillate was made alkaline and evaporated to dryness. The residue was dissolved in ether which contained 3% formic acid. The ether solution was analysed by gas chromatography. The temperature-programmed separation of acids was effected on a column packed with *Chromosorb* 101. Quantitation was based on the calibration curve obtained with butyric acid internal standard. No preservative loss was observed in the concentration range commonly used in bakery practice.

The method was applied to preservative-containing bread samples.

It was observed that, due to evaporation and decomposition during the baking process, significant and marginal losses occurred in the calcium propionate and potassium sorbate contents, resp. The method allows for the determination of the calcium propionate and potassium sorbate content of preservative-containing bakery products with an average relative error of ± 4.6 and $\pm 2.9\%$, resp.

Preservatives and other aging inhibitors are added to bread and other bakery products to extend their shelf-life. Their use is licensed by the health authorities. At present potassium sorbate and calcium propionate together (maximum 0.3% relative to flour) or potassium sorbate alone (0.2% relative to flour) are licensed in Hungary as bakery preservatives.

The prerequisite of the increased production of preservative-containing bakery products is the availability of suitable quality control methods. The development of such a method is the aim of our previous potassium sorbate determination methods (PÁLOSI-SZÁNTHÓ *et al.*, 1978) as well as of the present paper.

A gas-chromatographic method is presented here for the simultaneous quantitative determination of calcium propionate and potassium sorbate.

While there are many publications on the determination of either propionic or sorbic acid alone, only a few deal with the determination of the two acids simultaneously.

GRAVELAND (1972) used ether containing 3% orthophosphoric acid for the extraction of the preservatives. Gas chromatography was used for their separation and quantitation.

MEUSER and KLINGLER (1977) developed a special steam distillation apparatus for the extraction of preservatives from bread. The propionic acid content of the distillate was determined by gas chromatography, sorbic acid was measured by colorimetry.

While a number of analytical possibilities exist for the determination of sorbic acid present in bakery products (colorimetry, spectrophotometry, gas chromatography, thin-layer chromatography), only efficient separation methods can be used for the determination of propionic acid because acetic acid formed during the fermentation process disturbes the determination.

Due to the volatility of propionic acid, the separation method of choice is gas chromatography. Therefore, a gas-chromatographic method has been developed for the simultaneous determination of the two acids.

Polyesters on inert supports (HRIVNÁK, 1970; OTTENSTEIN & BARTLEY, 1971; GRAVELAND, 1972) and porous polymer packings (ACKMAN, 1972; JONES, 1973; DU PREEZ & LATEGAN, 1976) are both used for the determination of volatile, low fatty acids.

Apart from the stationary phase the column material also influences the separation and the reproducibility of the quantitative determinations. The polar acid molecules have a strong tendency for adsorption on the wall of the column.

In order to eliminate the adsorption phenomenon, ACKMAN (1972) used a carrier gas saturated by passing it over 98% formic acid. The formic acid thus introduced blocked the active centres on the surface of the stainless steel column. OTTENSTEIN and BARTLEY (1971) used phosphoric acid to treat the support and the column wall. They also proposed the use of the less polar glass column. DU PREEZ and LATEGAN (1976) used a 20% formic acid solution of the sample to suppress the adsorption of the organic acids.

1. Materials and methods

1.1. Chemicals

$MgSO_4 \cdot 7\ H_2O$, analytical grade
D-tartaric acid, analytical grade
formic acid, 98%, analytical grade
diethyl ether, analytical grade
Chromosorb 101; 80/100 mesh, *Johns-Manville*
n-butyric acid, analytical grade
potassium sorbate, analytical grade, *Merck*
calcium propionate, pract. grade, *Fluka*

1.2. Equipment

Specially constructed steam distillation apparatus (PETRÓ-TURZA, 1973; PÁLOSI-SZÁNTHÓ *et al.*, 1978)

Rotadest vacuum evaporator

Jeol 1100 gas chromatograph

TR 2215 A digital integrator

1.3. Composition of the samples tested

Sample No 1.:	wheat flour, BL 80	100.00 g
	calcium propionate	0.15 g
	potassium sorbate	0.15 g
	VX Trocken (emulsifier)	2.00 g
	water, bakery yeast, salt, saccharose, fat	
Sample No 2.:	wheat flour, BL 80	100.00 g
	calcium propionate	0.10 g
	potassium sorbate	0.20 g
	Rheopan (emulsifier)	1.25 g
	water, bakery yeast, salt saccharose, fat	
Sample No 3.:	wheat flour, BL 80	100.00 g
	calcium propionate	0.10 g
	potassium sorbate	0.20 g
	Tegomuls DW (emulsifier)	0.40 g
	water, bakery yeast, salt.	

1.4. Preparation of the bread samples

Only the inner part of bread loaves was used for the samples. Bread freed from its crust was cut, spread on filter paper, dried at ambient temperature for 24 h, and was ground by a coffee grinder. Bread crumbs thus obtained were stored in a ground stoppered glass jar.

1.5. Extraction of preservatives from bread

10 g air dried bread crumbs were weighed into the flask of the special steam distillation apparatus (PETRÓ-TURZA, 1973; PÁLOSI-SZÁNTHÓ *et al.*, 1978) along with 5 g D-tartaric acid, 30 g $MgSO_4 \cdot 7\ H_2O$. The flask was filled up to half of its capacity with distilled water. The apparatus was assembled and the distillation started. 200 cm^3 distillate were collected. It took about 30 min to distill the 200-cm^3 fraction.

2. Results and discussion

2.1. Extraction of preservatives from bread samples

Extraction of the calcium propionate and potassium sorbate preservatives was attempted first by the phosphoric acid – ether method as proposed by GRAVELAND (1972). Due to the fat content of the bread samples, a muddy solution was obtained, besides the recovery of potassium sorbate was not quantitative.

Next the extraction was attempted by using 3% formic acid instead of phosphoric acid. The results were not satisfactory either. Therefore, direct solvent extraction was abandoned in favour of steam distillation.

Since potassium sorbate cannot be recovered quantitatively by the conventional steam distillation apparatuses, a special steam distillation set-up, developed earlier for similar purposes, was used (PETRÓ-TURZA, 1973; PÁLOSI-SZÁNTHÓ *et al.*, 1978) the volatile acid content of the samples was liberated by tartaric acid, and magnesium sulphate (a salting-out agent). Distillation was carried out as described in Section 1.5. The distillate was made alkaline and evaporated to dryness in a *Rotadest*, in vacuum (max. evaporation temperature 40 °C). This step has to be included since propionic acid is soluble in both ether and water, so it cannot be directly transferred from water to an organic solvent, at least in a quantitative manner.

At first the ether containing phosphoric acid by GRAVELAND (1972) was used to liberate the volatile acids from their sodium salts in the alkaline residue. However, the gas-chromatographic determinations were not reproducible. The poor reproducibility is believed to be caused by the gel-like sodium phosphates which adsorb various amounts of the sorbic and propionic acids.

At last formic acid proved the most satisfactory agent for the liberation of the volatile acids.

The evaporation residue was dissolved in ether which contained 3% formic acid. The ether solution was reduced to 2 ml by flowing N_2 gas. This solution was used for the gas-chromatographic separation and quantitative determination of the acids.

2.2. Gas-chromatographic separation and quantitative determination of propionic and sorbic acids

Gas-chromatographic separation conditions were determined by using a 3% formic acid solution of the volatile acids studied. Separations were carried out by a *Jeol* 1100 gas chromatograph, equipped with a *FID* detector and a *TR* 2215 A digital integrator.

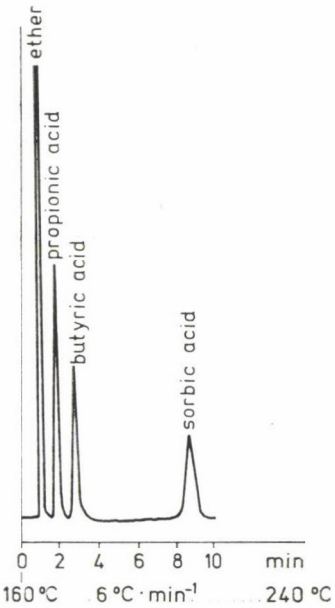

Fig. 1. Gas chromatogram of 5 μl of a model mixture of propionic (5 mg cm^{-3}), butyric (4 mg cm^{-3}) and sorbic acids (5 mg cm^{-3}). Solvent: 3% formic acid in ether. Attenuator: 128. Range: 10^{-10}. Column: 2.54 m × 2 mm I.D., aluminium tube packed with Chromosorb 101; 80/100 mesh. N_2 carrier flow rate: 40 cm^3 · min^{-1}. Injector and detector block temperature: 270 °C

Separations were effected on a 2.54 m long, 2 mm I.D. aluminium column packed with *Chromosorb* 101. The best separations were achieved by temperature programing at 6 °C min^{-1} from 160 °C to 230 °C. Nitrogen was used as carrier gas at a flow rate of 40 cm^3 min^{-1}. The injector and detector blocks were maintained at 270 °C.

2.2.1. Determination of the calibration curves. Once satisfactory separation of the two acids was achieved, the calibration curves used for the quantitative analysis were also obtained. In order to eliminate possible sources of occasional material losses 10, 15, 20 and 25 mg of calcium propionate and potassium sorbate were added to 10 g preservative-free air-dried bread crumbs. 8 mg butyric acid were also included in the calibration samples as an internal standard. Three parallel distillations were made at each concentration and chromatograms were recorded in triplicate as mentioned before. The extent of separation is shown in Fig. 1.

Using the integrator counts the ratios:

$$\frac{\textit{counts of propionic acid peak}}{\textit{counts of butyric acid peak}} \qquad \frac{P}{B}$$

and

$$\frac{\textit{counts of sorbic acid peak}}{\textit{counts of butyric acid peak}} \qquad \frac{S}{B}$$

were calculated.

With these ratios and the corresponding amounts of propionic and sorbic acid injected into the column (mg) the respective regression equations were determined. The following equations were obtained for calcium propionate:

$$y = 161.812x - 50.971 \qquad r = 0.9972$$

for potassium sorbate:

$$y = 116.822x + 5.502 \qquad r = 0.9985$$

where y: is the calcium propionate content and the potassium sorbate content resp. (mg%)

x: is the respective count ratio $\left(\frac{P}{B}; \frac{S}{B}\right)$

The unusually high intercepts in the regression equations indicate that there is a deviation from linearity at small x values. x is close to zero if either the peak area of the acid in question is zero or the peak area of the butyric acid internal standard is infinitely large. Since butyric acid is an internal standard added to the sample at an appropriate concentration, its value cannot cause $x = 0$.

In order to avoid $x = 0$ caused by too small solute peak areas, the amounts have to be selected in such a manner as to yield comparative peak areas for both the solute and the internal standard. This condition is further substantiated by the extent of inaccuracy introduced by the integration of small peaks. (In our case the ratios were in the 0.8–2.0 range.)

The high correlation coefficient values indicate the good correlation of the measured quantities.

Using the regression equations and the amounts of solutes added to the samples, the recovery rates were calculated as mg% along with the mg% standard deviations and the coefficients of variation at various concentration levels. The coefficients of variation were tested by the *Bartlett* test. The t test was used to test the added and found mg% values (VINCZE, 1968). The data relating to calcium propionate and potassium sorbate are shown in Tables 1 and 2, resp.

The calculated 95% confidence level χ^2 values are much lower than those listed in the tables indicating that the coefficients of variations are not significantly different.

Table 1

Recovery of calcium propionate added to air-dry bread crumbs

Ca-propionate added (mg%)	found	Average (mg%)	Standard deviation (mg%)	Coefficient of variation	$t_{calc.}$
100.00	105.99 102.75 101.13 102.75 104.37 102.75 99.52 97.90 99.52	101.85	2.57	2.53	2.159
150.00	148.06 146.44 149.68 152.91 149.68 152.91 149.68 151.30 152.91	150.40	2.30	1.53	0.521
200.00	199.84 193.37 198.22 193.37 196.60 194.98 194.98 191.75 201.46	196.06	3.24	1.62	3.648++
250.00	246.76 253.24 256.47 256.47 254.85 253.24 256.47 256.47 253.24	255.13	4.91	1.93	3.132+

Bartlett test: $\chi^2_{calc.} = 2.597 < \chi^2_{\alpha=0.05} = 7.815$

$^{+}\ t \geqq t_{0.05}$
$^{++}\ t \geqq t_{0.01}$

Table 2

Recovery of potassium sorbate added to air-dry bread crumbs

K-sorbate (mg%) added	found	Average (mg%)	Standard deviation (mg%)	Coefficient of variation	$t_{calc.}$
100.00	102.46 96.62 97.79 98.96 103.63 107.14 103.63 102.46 104.80	101.94	3.46	3.39	1.682
150.00	145.69 151.26 146.58 151.53 148.02 148.02 149.19 153.87 156.20	150.04	3.51	2.34	0.034
200.00	191.25 194.76 201.76 202.93 188.91 194.76 199.43 200.60 197.09	196.80	4.81	2.45	1.996
250.00	234.47 249.66 260.18 255.50 249.66 249.66 259.01 260.18 260.18	253.17	8.47	3.34	1.123

Bartlett test: $\chi^2_{calc.} = 1.831 < \chi^2_{\alpha=0.05} = 7.815$

The determination of calcium propionate could be characterised by a mean coefficient of 1.90% while that of the potassium sorbate of 2.88%. No significant difference was found between added and found potassium sorbate at any of the concentrations. Thus, it could be concluded that potassium sorbate can be recovered quantitatively from the samples. In the case of calcium propionate, significant deviations were obtained between the added and found values at the 200 and 250 mg% levels.

The deviations are mostly due to the extremely small standard deviations. Since the maximum permitted concentration of calcium propionate in bread is 150 mg%, and the added and found acid values did not differ significantly, the repetition of the measurements was not deemed necessary.

2.2.2. Examination of the applicability of the method developed. In order to test the technique developed with known preservative concentrations in the baked bread, experimental loaves were prepared in the BAKERY RESEARCH INSTITUTE.

The aim of these tests was also the determination of the effects of the various bakery additives: bakery yeast, salt, saccharose, fat, emulsifiers, *etc.*

The air-dried experimental loaves were subjected to the procedure developed here. Their moisture content was also determined in order to calculate the results on a dry-weigth basis, too. The composition of the experimental loaves is shown in Section 1.3.

The extraction and determination of the preservatives was carried out as described above. Both the distillation and the gas-chromatographic measurements were carried out in triplicate. The results relating to calcium propionate and potassium sorbate are shown in Tables 3 and 4, resp.

It can be seen that there is a smaller or greater, but always significant difference between measured and calculated values.

According to Section 2.2.1. these losses are not due to the analytical method, rather they are caused by evaporation and heat decomposition during the baking process. This conclusion is further substantiated by the fact that the losses of the more volatile propionic acid are larger than those of sorbic acid.

A temperature gradient exists in the loaves during baking, so the extent of decomposition and evaporation is different at different layers of the loaves. It follows that the distribution of the more volatile propionic acid in the sample is less homogeneous than that of sorbic acid.

This is the reason why the coefficients of variation were so different: $\pm 4.60\%$ for the calcium propionate (much higher than before) and $\pm 2.86\%$ for the potassium sorbate (practically identical with the previous value).

These findings are in agreement with those of MEUSER and KLINGLER (1977) who studied the effects of baking parameters upon the sorbic and propionic acid contents of the loaves. They concluded that during baking some

Table 3

Results obtained with test loaves containing known amounts of Ca-propionate

No. Sample	Ca-propionate added+ (mg%)	Ca-propionate found+ (mg%)	Average (mg%)	Standard deviation (mg%)	Coefficient of variation	$t_{calc.}$
1	177.36	103.96 107.52 107.52 118.18 119.95 114.62 103.96 109.30 109.30	110.48	5.83	5.28	34.42****
2	110.41	78.65 83.95 83.95 80.42 82.18 80.42 78.65 78.65 80.42	80.81	2.12	2.62	41.89****
3	113.53	81.37 77.71 86.84 74.05 74.05 86.84 77.71 79.54 79.54	79.74	4.70	5.89	21.57****

Bartlett test: $\chi^2_{calc.} = 4.724 < \chi^2_{\alpha=0.05} = 5.991$

+ relative to dry material content
**** $t \gg t_{0.001}$

Table 4

Results obtained with test loaves containing known amounts of K-sorbate

No. Sample	K-sorbate added+ (mg%)	K-sorbate found+ (mg%)	Average (mg%)	Standard deviation (mg%)	Coefficient of variation	$t_{calc.}$
1	177.36	175.38 174.10 172.82 170.25 176.67 172.82 179.23 172.82 175.38	174.39	2.62	1.65	3.401**
2	220.82	203.77 199.94 212.71 215.25 221.63 213.97 203.77 202.49 205.05	208.73	7.00	3.66	5.180****
3	226.92	224.71 231.94 225.34 217.42 221.38 210.82 214.78 220.07 216.10	220.18	6.39	3.28	3.164*

Bartlett test: $\chi^2_{calc.} = 4.597 < \chi^2_{\alpha=0.05} = 5.991$

\+ relative to dry material content
* $t \geqq t_{0.05}$
** $t \geqq t_{0.01}$
**** $t \geqslant t_{0.001}$

of these acids leave the loaves along with the water vapour in a manner similar to steam distillation. They found that the baking loss during the 35 min long baking period was 12–15%, that of the propionic acid was 35–40%, while that of the sorbic acid was only 12–15%.

The magnitude of loss depends on the surface area of the loaf, baking temperature and time, concentration of the preservatives, *etc.* Therefore, the amount of preservative actually used in the making of bread can only be estimated by the examination of the final products. It would be appropriate for the health control authorities to set limits for the preservative contents not only relative to flour but also, relative to the final product. The possibility for their doing so has become real by the development of the proper analytical method described in this paper.

*

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HEAT DESTRUCTION OF CLOSTRIDIUM BOTULINUM SPORES AND THE EFFECT OF EDTA THEREUPON

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Density distribution of spore populations of *Clostridium botulinum* 33A heat treated in the presence and absence of EDTA were determined by centrifugation in Renografin density gradient. In case of heat treatment in EDTA-free medium, density of cells was increasingly shifted towards lower values by increasing heat treatment. Density and refractivity of spores were more drastically reduced by heat treatment in the presence of 0.02 *M* EDTA than in its absence. Ca-DPA was released from spores as the result of heat treatment. In the presence of EDTA, Ca was removed from Ca-DPA, leaving the dipicolinic acid in the free acid form. Furthermore, in the presence of EDTA, the heat resistance of spores was substantially decreased. The observed effects of EDTA are likely to be due to the removal of divalent cations which might play an important role in membrane stability.

When bacterial spores are subjected to heating, a variety of breakdown processes occur (HUNNELL & ORDAL, 1961; GRECZ & TANG, 1970) resulting in losses of spore constituents particularly calcium dipicolinic acid (Ca-DPA), an abundant cytoplasmic constituent of spores (LEANZ & GILVARG, 1973). Loss of Ca-DPA during heating could be generally correlated with loss of spore viability under conditions when thermal injury was drastically varied by changes in pH of the heating medium (GRECZ *et al.*, 1972b) or by selection of individual strains of widely different basic heat resistance (GRECZ & TANG, 1970). Presumably the observed losses in spore constituents occur because of thermal damage to the membrane and to membrane permeability; in fact, electron microscopy revealed disruption of membrane integrity and blistering of the membrane in spores subjected to lethal heating (GRECZ *et al.*, 1972a). There is ample evidence that divalent ions, particularly calcium play a role in the heat stability of spores (SUGIYAMA, 1951; GRELET, 1952; AMAHA & ORDAL, 1957; BLACK *et al.*, 1962). If loss of divalent ions is indeed responsible for the loss of spore stability then it would be expected that heat treatment in presence of a metal chelator such as ethylene-diamine-tetraacetate would enhance the rate of loss of spore constituents during heating. Furthermore, since spore cytoplasmic constituents, particularly Ca-DPA contribute to the high density of spores (HALVORSON & SWANSON, 1969), it would be expected that heat-induced loss of cytoplasmic spore constituents would cause a commensurate decrease in density of the spores. The process of loss of density during heating

should in turn be accompanied by corresponding losses in spore viability (GRECZ & TANG, 1970).

From this point of view, the present study was concerned with the heat-induced changes in the spores including changes in density, the associated changes in spore viability, and the effect of ethylene-diamine-tetraacetate on these changes under conditions of mildly lethal, lethal, and supra-lethal heating.

1. Materials and methods

1.1. Production, harvesting and cleaning of spores

Spores of *Clostridium botulinum* strain 33A were produced by incubation for 4 days at 30 °C in TPT sporulation broth consisting of 5% tripticase (11921 BBL), 0.5% peptone (DIFCO B118) and 0.1% sodium-thioglycollate (FISHER S-442).

The spores were harvested by centrifugation at 11,500 g in *Sorvall* RC-2 centrifuge for 10 minutes.

Spores were cleaned to remove adhering vegetative sporangia by sequential incubation at 30 °C in 0.25 cm^3 each of filter-sterilized lysozyme (1.25 mg cm^{-3} for 1.5 h), pronase (2.5 mg cm^{-3} for 1.5 h) and 5% sodium-lauryl-sulfate for 0.5 h. Then spores were twice washed with 10 cm^{-3} of sterile distilled water, and stored in distilled water at 2–4 °C until needed, but not longer than one week.

1.2. Heat treatment

Cleaned resting spores were suspended in distilled water. Aliquots of this stock suspension were distributed into small glass vials and EDTA-Na_2 was added to a final concentration of 20 *mM*. EDTA-free suspensions of the same cell count served as controls. The samples were heated at 90 °C for 5, 10 and 30 minutes, as well as at 121 °C for 35 minutes, resp.

1.3. Viable counts

Viable cell counts were made by preparing serial decimal dilutions in 0.067 *M* phosphate buffer (pH 7.0) and subculturing 1.0 cm^3 samples in triplicate tubes of WYNNE's medium (WYNNE *et al.*, 1955) plus 0.75% agar (ELLER *et al.*, 1967). The solidified medium was stratified with about 2 cm of WYNNE's agar and was incubated at 30 °C up to 5 days.

1.4. Measurements of the density distributions of spores

Isopicnic density gradient centrifugation has been successfully applied in some laboratories for determination of the distribution of cells by density (TAMIR & GILVARG, 1966; ROGACHEVSKII & ZVIAGINTSEV, 1973; ZVIAGINTSEV & ROGACHEVSKII, 1973). A similar determination of density distribution can be made on bacterial spore populations, provided that the chosen centrifugation medium affords the specific gradient range needed for the separation of spores. Based on the publication by TAMIR & GILVARG (1966) *Renografin*-76 X-ray contrast material (SQUIBB and SONS INC., Princeton, N. J.) was chosen by us; this material was successfully applied by the above authors to separate bacterial spores and vegetative cells as well as to fractionate spore populations by density. *Renografin* is a water solution composed of 66% methylglucomin-3,5-diacetamido-2,4,6-triiodobenzoate, 8% sodium-3,5-diacetamido-2,4,6-triiodobenzoate, 0.32% Na-citrate and 0.04% di-sodium-ethylene-dinitrilotetra-acetate. Exponential *Renografin* gradients were prepared using the method developed in our laboratory by DEMET (1976).

After heat treatment the spore suspension was stratified on top of the *Renografin* density gradient as specified by TAMIR and GILVARG (1966). Next, the plastic (polyallomer) tubes containing the gradients were centrifuged at 15 000 rpm for 30 minutes in an SW-27 type rotor at 4 °C in a *Beckman Spinco-L* centrifuge. After centrifugation the bottom of the plastic tubes was pierced by a syringe and the drip-out content of the tubes was collected in 1.5 cm^3 fractions by means of the automatic fraction collector, *Gilson Linear Fractionator* Model VL. A microscopic preparation was made from each fraction and the total cell number of the fractions was determined by phase-contrast microscope using the slide chamber method (VAS, 1962).

Renografin shows a very strong light absorption in the UV-range with an absorption maximum of 260 nm. Since there is a linear correlation between the density and absorbance of *Renografin* solutions (TAMIR & GILVARG, 1966), the density of the fractions could be easily determined therefore, by the spectrophotometric method after proper (10^4-fold) dilution of the samples.

2. Results

Figure 1 summarizes our data on spore distribution in *Renografin* gradients after heating in presence or absence of 0.02 *M* EDTA-Na_2. The per cent of spores found in each of the density ranges was calculated on the basis of microscopic spore counts in the respective fractions collected after heating from the *Renografin* gradients. For illustration purposes, the fractions in Fig. 1 were divided into density ranges of identical width (0.02 g cm^{-3}), and the

weighted averages of the spores in these density ranges were calculated and plotted in Fig. 1. In addition, the mean density of the various spore populations subjected to a particular experimental treatment were calculated. The mean densities for each category of spores are given in the form of numerical values and indicated by arrows in Fig. 1.

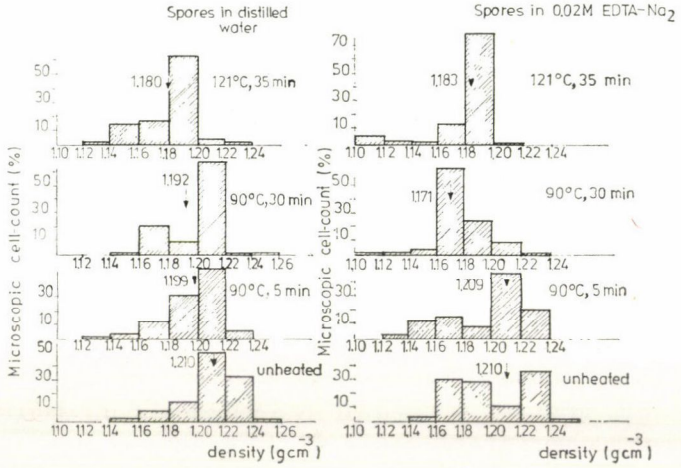

Fig. 1. Density distribution in Renografin gradient of spore populations of *C. botulinum* 33A as a function of heat treatment in presence or absence of 0.02 *M* EDTA-Na_2

The mean density of the spore population before heating was 1.210 g cm^{-3} although ther ewere distinc tdifferences in the density profiles of spores suspended in distilled water and those in 0.02 *M* EDTA. Whereas unheated spores in distilled water showed a skewed bell-shaped density distribution profile the spores in EDTA showed a rather irregular distribution with a large proportion of spores having relatively low densities (1.16–1.20 g cm^{-3}). This seems to suggest that EDTA may have caused some immediate changes in the densities of the spores even in absence of heating, although these changes did not alter the calculated overall numerical value of the mean density of the spore population.

Exposure to heat invariably led to a decrease in the mean density values so that after heating the density distribution profiles in Fig. 1 shifted toward lower values. Three levels of heating were applied, *viz.*, mildly lethal (90 °C for 5 min), lethal (90 °C for 30 min), and supra-lethal (121 °C for 35 min). As compared with unheated controls, spores heated in distilled water at 90 °C showed a decrease in mean density values by $11 \cdot 10^{-3}$ g cm^{-3} and by $18 \cdot 10^{-3}$ g cm^{-3} after heating for 5 and 30 min, resp. On the other hand, in EDTA after similar heating for 5 and 30 min, the mean density values decreased by $1 \cdot 10^{-3}$ g cm^{-3} and by $39 \cdot 10^{-3}$ g cm^{-3}, resp. It is clear then that the presence

of EDTA during heating at 90 °C for 30 min caused a 2-fold greater decrease in the value of mean density as compared with spores heated in water.

In the case of supra-lethal heating (121 °C for 35 min), a further decrease in the value of mean density of spores was noted. However, in this case the final mean density values of spores heated in presence and absence of EDTA were found to be practically identical. It should be pointed out however, that the spore population heated in presence of EDTA showed again a rather irregular sedimentation distribution in that some spores showed conspicuously low densities (1.10–1.12 g cm^{-3}); these low density fractions were absent from sedimentation profiles of spores heated in distilled water. Interestingly the low density fractions of spores heated in EDTA did not materially affect the mean density computed for this spore population; essentially identical mean density values were obtained for spores heated in distilled water (1.180 g cm^{-3}) as for spores heated in EDTA (1.183 g cm^{-3}).

In addition to changes in density described above, heating also caused decrease in optical density of the spores as shown in Fig. 2. Using direct photometry, a decrease in absorbance was detected already after 5 min of heating at 90 °C, and this decrease was even more extensive after supra-lethal heating at 121 °C for 35 min. In all cases, the spores lost optical density to a larger extent in presence of 0.02 *M* EDTA as compared to spores suspended in distilled water. Interestingly, EDTA caused loss of optical density even in control spore populations, *i.e.* those not subjected to heating. Along the same line, in comparing microscopic preparations from non-heated and heated spores some decrease in phase-contrast refractivity could be detected visually after heating, although no further attempt was made to measure quantitatively these microscopic changes.

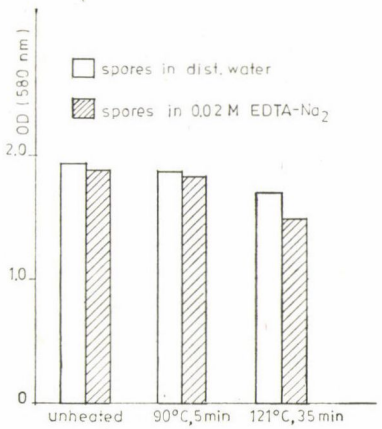

Fig. 2. Changes in absorbance at 580 nm of spore suspensions of *C. botulinum* 33A with identical cell counts (approximately 10^9 cm^{-3}) as a function of heat treatment in presence and in absence of 0.02 *M* EDTA

As shown in Fig. 3, UV-absorption spectra of exudates after heating of spores at 121 °C for 35 min were also distinctly different in the two heating media, *i.e.* in distilled water or in 0.02 *M* EDTA. When the spores were heated in distilled water, the UV absorption spectrum of the spore exudate (Fig. 3A, solid line) was essentially identical with that of the pure Ca(II)-DPA chelate (Fig. 4, solid line). Furthermore, when pure Ca-DPA was added to distilled water and similarly heated a typical spectrum of Ca-DPA chelate was obtained (Fig. 3A, dashed line) similar to the spectrum of the exudate from spores heated in distilled water (Fig. 3A, solid line). On the other hand, when spores were heated in aqueous 0.02 *M* EDTA, the UV-spectrum of the spore exudate (Fig. 3B, solid line) closely resembled that of free dipicolinic acid (Fig. 4, dashed line). Furthermore when pure Ca-DPA was added to 0.02 *M* EDTA and similarly heated, the resultant UV-spectrum (Fig. 3B, dashed line) was essentially identical with that of pure dipicolinic acid (Fig. 4, dashed line).

These observations suggest that the very strong chelating action of EDTA has withdrawn the calcium and left the DPA in the acid form, regardless whether pure Ca-DPA was added to the suspension or whether the Ca-DPA was lost from the spores as the result of heating. These UV absorption spectra of spore exudates confirm our previous observations that the dominant UV-absorbing component of the spore exudate was Ca-DPA (GRECZ & TANG, 1970; GRECZ & JAW, 1972).

Since EDTA had a distinct effect on the density of spores as well as on the release of calcium and DPA during heating, it was next of interest to

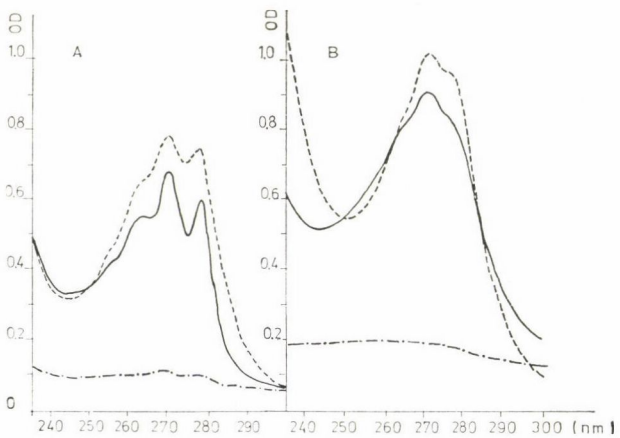

Fig. 3. Ultraviolet absorption spectra of exudates from *C. botulinum* 33A after heating at 121 °C for 35 min (solid line) in distilled water (A) or in 0.02 *M* EDTA (B). The dotted line in the bottom of (A) and (B) gives the absorption spectra of the respective suspending media when the spores were not subjected to heating (unheated spores). For comparison this figure gives the spectra of 0.2 *mM* Ca-dipicolinate in both suspending media used in this study, in distilled water (dashed line in A) and in 0.02 *M* aqueous EDTA (dashed line in B)

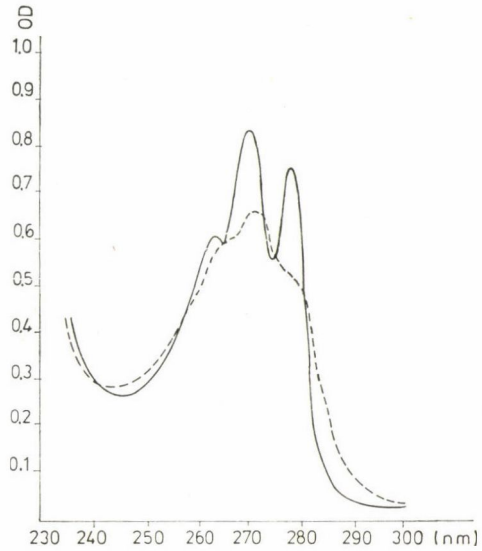

Fig. 4. Ultraviolet absorption spectra of 0.155 *mM* aqueous solutions at pH 7 of dipicolinic acid (dotted line), and calcium dipicolinate (solid line)

study the effect of EDTA on the lethal effect of heat on the spores. In this experiment relatively mild heat treatments were applied (90 °C for 5 and 10 min) so as to obtain measurable survival rates. The results presented in Table 1

Table 1

Effect of EDTA in the suspending medium on the heat inactivation of spores of Clostridium botulinum *33A*

Time of heating at 90 °C (minutes)	% Survival[a]		Factor of enhancement of lethality by EDTA
	in distilled water	in 0.02 *M* EDTA	
5	72.2 ± 27.0	10.9 ± 2.5	6.6
10	32.6 ± 10.7	5.6 ± 3.0	5.8

a) Viable counts of spores after heating were determined using WYNNE's Broth (WYNNE *et al.*, 1955) plus 0.75% agar in oval Pri culture tubes. Incubation was at 30 °C for 5 days. Mean values $\pm$ confidence intervals from quadruplicate counts, $P \leq 5\%$, $d_f = 3$

clearly indicate that 0.02 *M* EDTA has substantially increased the rate of termal inactivation of the spores. Under our conditions, heating at 90 °C for 5 min caused inactivation of some 30% of the spores in distilled water as compared to some 90% in EDTA, *i.e.* a 6.5-fold enhancement of the lethal effect due to EDTA. Similarly, after 10 min of heating the enhancement of the lethal effect by EDTA was about 6-fold, *i.e.* from approximately 70% in distilled water to some 94% in EDTA.

3. Discussion

The present study for the first time reports density values for anaerobic spores, particularly those of *Clostridium botulinum*. In the past only spores of aerobic organisms have been investigated for their density and the values in the literature range from about 1.2 g cm^{-3} to 1.39 g cm^{-3} (Table 2).

The density values for *Bacillus* spores of approximately 1.2 g cm^{-3} reported by early investigators (reviewed by LAMANNA *et al.*, 1973), may be compared with 1.28 g cm^{-3} obtained more recently by BLACK and GERHARDT (1962) for resting spores of *B. cereus* T. On the other hand SICCARDI and his co-workers (1975) arrive at 1.390 g cm^{-3} for *B. subtilis* spores, which appear to form the most dense cells.

The method of isopicnic density gradient centrifugation in *Renografin* used in this study for determination of the density of spores was first employed by TAMIR and GILVARG (1966). The density value obtained by them was 1.305 g cm^{-3} for "normal" Ca-DPA containing spores of *B. megaterium*. The value of 1.210 g cm^{-3} determined for unheated spores of *C. botulinum* 33A overlaps with the low density values for aerobic spores reported in the early literature (LAMANNA *et al.*, 1973). Although somewhat lower than the values for *Bacillus* spores cited in the more recent literature, it is still higher than the densities of DPA-less spores or germinated spores of *B. cereus* shown in Table 2.

The great variability in the literature concerning density of spores as summarized in Table 1 is partly due to the substantially different experimental methods employed in the various laboratories. However, the values from individual laboratories obtained in each case by the same method are validly comparable and allow certain meaningful conclusions. From Table 2, density value responsible for the spore state is approximately 0.17 g cm^{-3}. This value can be approximated from the difference between "normal" Ca-DPA containing spores (1.28 g cm^{-3}) and the germinated spores (11.1 g cm^{-3}) of *B. cereus* T (BLACK & GERHARDT, 1962). The density of germinated spores is apparently identical or perhaps slightly lower than the values reported for vegetative cells. Furthermore, from Table 2 at least two factors responsible for the high density of spores may be broadly identified: (i) Ca-DPA, and (ii) factors other than Ca-DPA.

The role of Ca-DPA can be evaluated by comparing the densities of Ca-DPA containing (1.27 g cm^{-3}) and DPA-less (1.18 g cm^{-3}) spores of *B. cereus* T as determined by WISE and his co-workers (1967). Since the only known difference between these two types is the presence or absence of DPA, it appears that DPA (probably in the form of Ca-DPA) contributed 0.09 g cm^{-3}. Therefore, Ca-DPA in this case must have contributed 53% of the spore density, while factors other than Ca-DPA account for the remaining 47%. The

Table 2

Average densities of bacterial endospores and vegetative cells

Microorganisms	Density in g cm^{-3}						
	B. cereus T	*B. megaterium*	*B. subtilis*	*C. botulinum* 33A	*Azotobacter vinelandii*	*E. Coli*	Other microorganisms
"Normal" DPA-containing spores	1.28[c]; 1.27[f]	1.305[e]	1.115[a]; 1.37[d]; 1.39[l]; "heavy" 1.335[k] "light" 1.290[k]	1.210[m]	–	–	1.2[b]
DPA-less spores	1.18[f]	–	–	–	–	–	–
Heat killed spores	–	–	–	1.180[m]	–	–	–
Germinated spores	1.11[c]	–	1.350[l]; 1.2[k]	–	–	–	–
Vegetative cells	–	1.135[e]	1.105[a]; 1.17–1.24[l]; 1.136[h]; 1.152[h]; 1.110[i]; 1.131[l]	–	1.108[j]; cyst: 1.152[j]	1.094[a]; 1.16[e]	1.05–1.112[b,g]

References: a) RUFFILLI, 1933; b) LAMANNA *et al.*, 1973; c) BLACK & GERHARDT, 1962; d) LEWIS *et al.*, 1965; e) TAMIR & GILVARG, 1966; f) WISE *et al.*, 1967; g) FIHMAN, 1967; ROGACHEVSKII & ZVIAGINTSEV, 1973; ZVIAGINTSEV & ROGACHEVSKII, 1973; h) CAHN & FOX, 1968; i) HADDEN & NESTER, 1968; j) WETEGROWE & WYSS, 1974; k) DEAN & DOUTHIT, 1974; l) SICCARDI *et al.*, 1975; m) This study

major role of Ca-DPA in this respect is not surprising in view of the high density of chemically pure Ca-DPA (1.71 g cm^{-3}) according to TAMIR and GILVARG (1966), and the high concentration of this substance in spores. The Ca-DPA content per dry weight of spores can be estimated (assuming a 1 : 1 Ca : DPA ratio) as 11.7 % in *B. cereus* T (from the data of MURRELL and WARTH, 1965) and as 16.6 % in *C. botulinum* 33A (from the data of GRECZ and TANG, 1970).

DEAN and DOUTHIT (1974) presented evidence that a broad band of *B. subtilis* spores in continuous gradients of *Renografin* could be resolved into two major bands of spores, a heavy one at 1.335 g cm^{-3} and a light one at 1.290 g cm^{-3}. The heterogeneity of spore densities is not due to *Renografin* because removal of the chelating agents present in *Renografin* did not influence the density pattern of *B. subtilis* spores. Heavy spores had 30 % more dipicolinic acid than light spores. Light spores were more heat sensitive than heavy ones. The light spores may represent an immature stage of sporulation despite the fact that they are released from the sporangium.

The decrease in density of spores during heating is perhaps best explained in terms of loss of Ca-DPA. The materials lost from the spore of *C. botulinum* 33A during lethal heating were shown chromatographically to be primarily Ca-DPA with minor components due to other substances (GRECZ & JAW, 1972). At the instant of thermal death spores lose up to 70 % of their original DPA, and the remaining DPA continues to be released on further heating (GRECZ & TANG, 1970). However, the spores subjected to supra-lethal heating (density 1.17–1.19 g cm^{-3}) have most certainly lost all their Ca-DPA.

No definite information is available about the state of the DPA in the spores of the manner in which it is released on heating, although there is rather good evidence that the DPA is located within the innermost protoplast of the spores (LEANZ & GILVARG, 1973). From gravimetric studies it has been postulated that the spore interior is stabilized by a protective cement consisting of chelates of divalent metals with spore ligands; DPA and Ca play a major role in this model primarily due to their natural abundance in the spore. Charged and polar groups of cell molecules bind oppositely charged and polar groups in the "cementing" material; the resulting structure is of low polarity, low electrostatic charge, low chemical reactivity, and last but not least low hygroscopicity (GRECZ *et al.*, 1970). As shown in the present study this molecular arrangement is also associated with a high density, high optical density and high phase contrast refractivity. On lethal heating these spore properties are only partially lost. From Table 2 the residual density of heat killed spores – even after supra-lethal heating – remains equal to that of DPA-less spores, and the spores still exhibit a relatively high phase contrast refractivity.

The factors other than Ca-DPA which contribute some 47 % to the specific gravity of spores are still not definitely recognized and remain the

subject of much speculation. A contracted state of cortex such as proposed by LEWIS *et al.* (1960) may conceivably involve molecular condensation resulting in high density. The contractile cortex theory however has been seriously challenged. On the basis of some experimental evidence GOULD and DRING (1975) propose that an expanded – rather than contracted – cortex exerts osmotic pressure on the central protoplast. According to this model, the electronegative peptidoglycan biopolymer in combination with positively charged counterions functions as an osmoregulatory organelle. In this sense then the cortex would be expected to have a low rather than high density. This argument is essentially confirmed by available electron micrographs where the cortex invariably appears as a layer of low electron density; such light images are generally due to materials of low density.

An alternative view of possible relevance regarding the factors other than Ca-DPA is based on the observation that spore molecules are abundantly cross-linked by disulfide bonds. BLACK and GERHARDT (1962) proposed that molecular interpolymerization occurs in the spore analogous to that observed with thiolated gelatins. Such gels when oxidized gradually form insoluble heat stable gels that are reversible with reducing agents. A large reduction in spore protoplast volume is seen in electron micrographs during spore maturation; this was explained by ionic and electrolytic reordering of macromolecules, *i.e.* synaeresis (GERHARDT & MURRELL, 1978), a form of molecular condensation that conceivably may lead to increased density. Thiol compounds exist in the spores in the oxidized disulfide form, either dimerized or linked to protein molecules (SWERDLOW *et al.*, 1979). The disulfide-rich structures have been variously linked to heat resistance and metabolic dormancy of spores (VINTER, 1961; BLANKENSHIP & PALLANSCH, 1966; SCHMIT & BRODY, 1976).

Spores contain relatively high levels of NADH-linked disulfide reductase (SWERDLOW *et al.*, 1979) which seems to play a role in depolymerization of disulfide biopolymers during spore germination. Subsequently, depolymerized spore molecules undergo hydration which results in swelling and reduction of density during spore germination (PRENTICE *et al.*, 1972). In this case both, Ca-DPA and the factors other than Ca-DPA are either lost or changed so that germinated spores become essentially equal in density to vegetative cells. On the other hand, on lethal heating the enzymes in the spore are inactivated. Therefore, if molecular interpolymerization such as postulated by BLACK and GERHARDT (1962) is indeed involved in the increased density of spores, then heat-killed spores would not be depolymerized and therefore would retain relatively high density equal to that of DPA-less spores. This was in fact observed to be the case in the present study.

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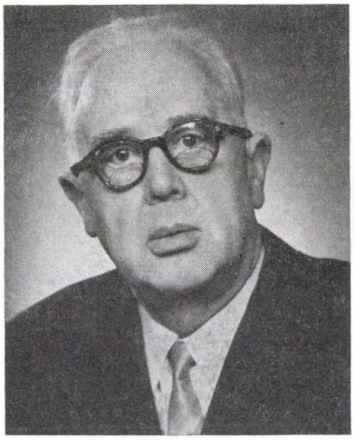
IN MEMORIAM P. SPANYÁR



The Editorial Board of *Acta Alimentaria* deeply regrets to announce that one of its founding members, Dr. PÁL SPANYÁR passed away on 29 April 1980 at the age of 77.

Born at Székelyudvarhely (Transylvania), Dr. SPANYÁR finished his university studies in the faculty of chemical engineering of the Technical University of Budapest in 1926. He spent the first ten years of his professional career at the Institute of Food Chemistry of the Technical University of Budapest and at the University of Budapest, working in the fields of food chemistry and clinical biochemistry. For the next 14 years, he played a leading role in the management of a number of firms of the Hungarian chemical and food industries dealing with as diverse products as dyes, plant protectants, essential oils, aroma products and canned foods. He founded several new factories and introduced the manufacture of many new products.

After such a multifarious career, the most prolific part of SPANYÁR's scientific life started thirty years ago when he was 47. For full three decades, he could dedicate himself to food research, concentrating on food chemistry and analysis. All these years he spent in his laboratory in the Buda hills as Head of the Analytical Chemistry Department of the Central Food Research Institute and of the latter's predecessor, the Institute for Research in Canning, Meat Packing and Refrigeration, respectively.

He made his mark as a scholar in the chemistry of foods, dealing specifically with the fundamentals of food smoking and smoke generation, with the analysis of ascorbic, benzoic and sorbic acids in foods, with the determination of capsaicin in paprika, with trace elements and aroma substances in foods. Stabilisation of the witamin C content of foods, as well as the mechanism of browning of food products were among the subject matters he was engaged in for a long time. The results of all this activity are contained in some 200 scientific publications and books and in a large number of industrial applications mainly in the Hungarian canning, refrigeration and meat industries.

Dr. SPANYÁR was a pioneer of Hungarian food standardisation. He was instrumental in the generation of more than a hundred standards.

He was the editor of "Élelmiszertudomány" (Food Science), the predecessor of *Acta Alimentaria* and founding member of the latter journal since 1971.

As a member of the Committee on Food Science of the Hungarian Academy of Sciences, Dr. SPANYÁR greatly contributed to the development of this

sector of Hungarian science. He also played an important role in the activities of the Hungarian Scientific Society for Food Industry.

Dr. SPANYÁR's death is a serious blow to food science and technology in this country. He will long be missed by his fellow scientists, his pupils and Hungarian science in general. He was an amiable person, leaving behind many friends and admirers.

He will be remembered by all those working in food research in Hungary and abroad.

Acta Alimentaria, Vol. 9 (4), pp. 305—312 (1980)

DETERMINATION OF THE AUTOLYSIS OF CHAMPAGNE YEAST BY USING ^{14}C-LABELLED YEAST

I. MOLNÁR, E. OURA and H. SUOMALAINEN

(Received 13 September 1979; accepted 5 January 1980)

The degree of autolysis of ^{14}C-labelled *Champagne Hautvillers* yeast was followed up at different temperatures of storage. A linear relationship was found between the length of the storage and the degree of autolysis. The rate of autolysis increased with raising the temperature of storage. Raising of the temperature by 10 °C was followed by a 6–7% increase in the rate of autolysis. Shaking up the yeast sediment at 20-day intervals raised the rate of autolysis by 1.5–4.2%.

During bottle fermentation of sparkling wine, aging lasts for 2 to 3 months. After the sugar content has been exhausted, the glycogen reserve is consumed and yeast cells enter a latent state, then the cells autolyse. The autolysis proceeds under the influence of environmental factors including the temperature of aging and the mode of contact between yeast cells and sparkling wine. A concentration gradient of components of cellular origin developing around yeast cells suppresses further autolysis. The concentration gradient and its depressive effect can be suspended by shaking up the sparkling wine.

1. Materials and methods

1.1. Microorganism

Champagne Hautvillers yeast was used throughout. It was maintained on slant malt agar.

1.2. Fermentation

A synthetic medium was used. Its pH was adjusted to 4.5 by adding 2*N* H_2SO_4. The medium was sterilized by autoclaving at 115 °C for 30 min. Fermentation was performed in an LKB BIOTEC fermentor of continuous operation, under anaerobic conditions. Fermentation temperature: 28 °C, pH: 4.5. Nitrogen gas, 60 l h^{-1}, presterilized by filtration, was driven through the liquid. The rate of medium inflow was 80 cm^3 h^{-1}; at the same time, 15 cm^3 h^{-1}

4 mCi D-(U-^{14}C) glucose was added to the fermentation juice. The developing CO_2 was bound by an alkaline and a subsequent acid washing. The overflowing liquid was collected in an ice-cooled vessel. The fermentation was finished and the suspension was cooled to 0 °C as the whole amount of glucose has been added. Then the labelled yeast cells were separated from the fermentation broth by cold centrifugation and the sedimented yeast was washed in ice-cold distilled water until the supernatant became free of radioactivity.

To determine ^{14}C activity, yeast was hydrolysed in 3 *N* HCl at 110 °C for 4 h, the hydrolysate was evaporated *in vacuo* and redissolved in distilled water. ^{14}C activity per cm^3 yeast was determined using a scintillation counter.

1.3. Preparation of sparkling wine samples containing labelled yeast

^{14}C-labelled yeast, 1.725 g l^{-1}, was suspended in sparkling wine of 0 °C containing 2 mg cm$^{3-1}$ NaF. To avoid CO_2 loss, the samples were dispensed in flasks and the flasks were stoppered at the same temperature. Bottles were then kept at 4, 10, 20, 30 and 40 °C, and the radioactivity of the already cell-free samples was measured at 10-day intervals. In each temperature group, some of the flasks were thoroughly shaken up at 20-day intervals, the other flasks were stored without shaking.

1.4. Radioactivity assay

To determine ^{14}C activity, an ULTROBETA 1210 Liquid Scintillation Counter (LKB Wallace) and the Packard Insta-Gel scintillation system were used. From the labelled samples to be tested, the cells were removed by centrifugation at 1000 g for 3 min. The efficiency of scintillation measurement was determined by external standardization. The activity has been defined in dpm units (disintegration per minute) *i.e.* by the number of radioactive disintegrations per minute.

2. Results and conclusions

During the autolysis of yeast cells, cellular components, including β-active ones, diffused into the champagne. The quantity of the released activity depended on the number of the autolysed cells, *i.e.* the increase in β-activity at the given conditions including temperature linearly related to the degree of autolysis. However, two disturbing factors had to be taken into account. One of these is that the β-activity incorporated in insoluble cell components was removed from the sparkling wine by centrifugation. Thus, the measured increase in β-activity was less than the real activity.

The other effect was more disturbing, *viz.* the labelled yeast cells may continue their metabolic activities in the sparkling wine, consequently, part of the measured β-activity will result from this metabolism, not from autolysis. The radiolabelling method is not reliable in measuring autolysis if autolysis is not the only way in which activity can leave the cell. To avoid loss of β-activity due to metabolic processes, we blocked the metabolism by adding NaF, an inhibitor that does not impair the cell membrane and does not accelerate autolysis in this way. We are not convinced that the increase in activity observed in the sparkling wine early during storage had derived from natural autolysis exclusively, therefore, the results of the first 10-day period were omitted. For the purposes of our experiments, the activity in this period is of no significance, since the effect of the autolysis of yeast needs a prolonged time to be manifested.

Results of measurements are given in Table 1 and Figs. 1, 2, 3, 4 and 5. The correlation coefficients obtained by statistical evaluation of the results as well as the constants of the regression equations are shown in Table 2.

Table 1

Changes in the β-activity of sparkling wine as an effect of storage with ^{14}C-labelled champagne yeast

Storage in days	^{14}C activity of champagne dpm cm^{-3} at									
	4 °C		10 °C		20 °C		30 °C		40 °C	
	unshaken	shaken	unshaken	shaken	unshaken	shaken	unshaken	shaken	unshaken	shaken
10	3 000	3 100	7 580	7 610	13 950	14 100	17 550	17 700	24 160	24 600
20	9 240	9 800	15 600	16 390	21 600	22 520	27 650	28 790	34 900	36 140
30	14 070	14 150	19 570	20 850	29 060	30 140	35 100	36 820	41 880	43 700
40	15 230	15 900	22 240	24 500	32 550	34 600	38 520	41 540	45 950	48 220
50	16 480	17 540	26 210	29 470	34 140	38 750	43 570	47 400	49 330	53 800
60	17 400	20 050	30 280	32 850	39 250	41 680	44 950	51 200	56 420	63 210
70	20 960	22 100	32 070	34 000	43 560	48 320	54 130	56 980	61 800	66 340
80	23 700	26 070	33 650	37 100	45 230	50 200	56 800	60 830	67 350	72 700
90	25 270	27 820	36 810	39 080	47 410	55 110	60 270	65 470	74 240	81 960
100	26 550	29 500	38 420	41 130	54 320	58 570	64 390	70 880	83 520	88 100
110	29 200	33 140	39 700	42 700	57 580	60 400	71 800	76 200	88 070	94 350
120	31 400	33 500	40 610	44 260	59 800	63 900	75 220	81 840	91 230	98 920

In our experiments, the autolysis was speeded up by raising the temperature of storage as well as by shaking up the yeast sediment at intervals. High temperature acts *via* acceleration of destructive processes in general while shaking destroys the circumcellular concentration gradient which, if present,

Table 2

Statistical evaluation of the radioactivity of cell-free sparkling wine

Conditions of storage	Correlation coefficient (*r*)	Linear regression coefficients	
		a	b
4 °C no shaking	0.987	4 520	228.2
4 °C shaking	0.991	4 520	258.7
10 °C no shaking	0.978	10 170	282.0
10 °C shaking	0.985	10 850	307.5
20 °C no shaking	0.990	14 480	390.1
20 °C shaking	0.993	15 200	430.0
30 °C no shaking	0.987	17 470	487.3
30 °C shaking	0.990	18 080	536.3
40 °C no shaking	0.993	21 070	597.4
40 °C shaking	0.987	21 520	657.1

slows down the release from cells. From the changes in activity, the real degree of autolysis was calculated for a 100-day period under the conditions indicated in Table 3. The calculation was performed as follows.

Each experimental sample contained yeast corresponding to 1.725 g dry matter of yeast origin. The activity of the labelled yeast was 90 600 dpm mg^{-1}. From the measured values, the changes in activity/100 days (Δ dpm)

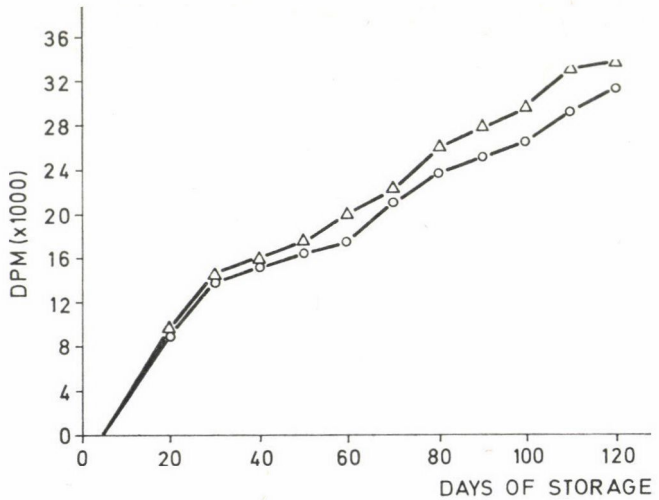


Fig. 1. Dynamics of the release of β-activity into sparkling wine containing ^{14}C-labelled *Ch. Hautvillers* champagne yeast during storage at 4 °C
–o–o– activity obtained without shaking up the yeast
–Δ–Δ– activity obtained in sparkling wine shaken up at 20-day intervals during storage

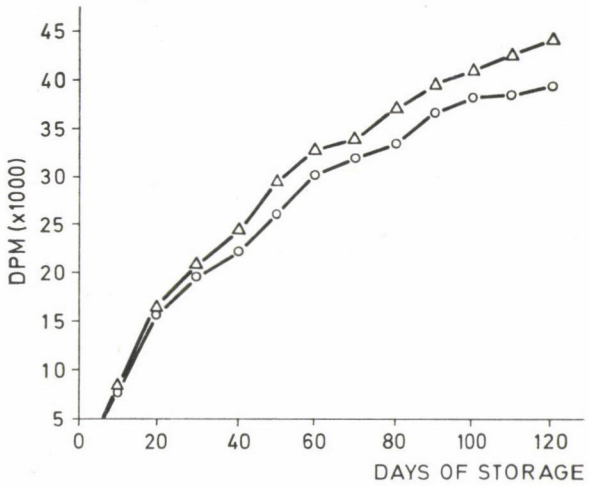


Fig. 2. Dynamics of the release of β-activity into sparkling wine containing ^{14}C-labelled *Ch. Hautvillers* champagne yeast during storage at 10 °C.
–o–o– activity obtained without shaking up the yeast
–Δ–Δ– activity obtained in sparkling wine shaken up at 20-day intervals during storage

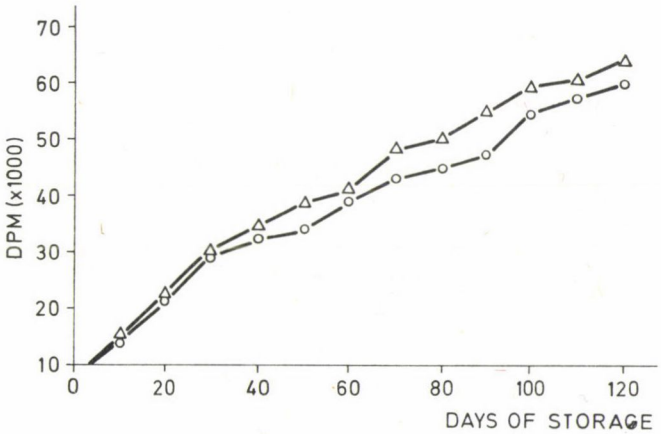


Fig. 3. Dynamics of the release of β-activity into sparkling wine containing ^{14}C-labelled *Ch. Hautvillers* champagne yeast during storage at 20 °C
–o–o– activity obtained without shaking up the yeast
–Δ–Δ– activity obtained in sparkling wine shaken up at 20-day intervals during storage

were estimated. Given the activity corresponding to a unit of yeast dry matter, we calculated the quantity of the autolysed yeast that had diffused into the sparkling wine. The degree of autolysis was calculated from the ratio of autolysed to initial amount of yeast and expressed in per cent. The results are presented in Table 3.

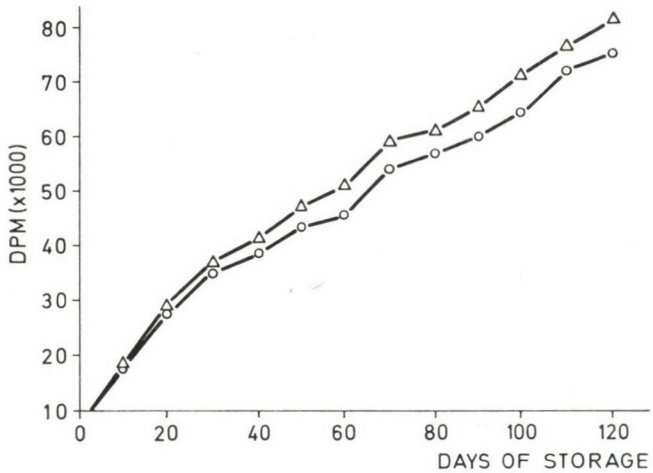


Fig. 4. Dynamics of the release of β-activity into sparkling wine containing ^{14}C-labelled *Ch. Hautvillers* champagne yeast during storage at 30 °C
–o–o– activity obtained without shaking up the yeast
–Δ–Δ– activity obtained in sparkling wine shaken up at 20-day intervals during storage

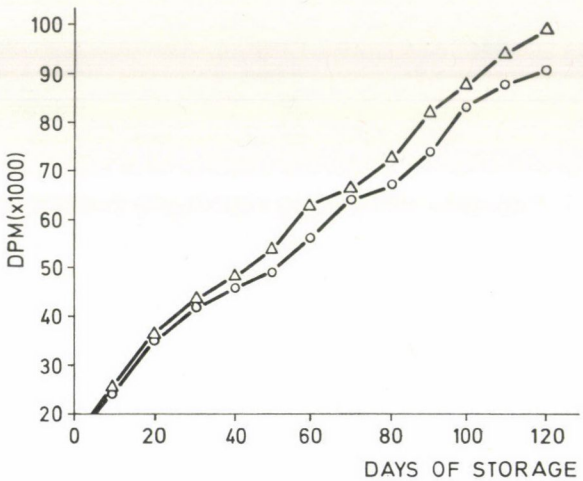


Fig. 5. Dynamics of the release of β-activity into sparkling wine containing ^{14}C-labelled *Ch. Hautvillers* champagne yeast during storage at 40 °C
–o–o– activity obtained without shaking up the yeast
–Δ–Δ– activity obtained in sparkling wine shaken up at 20-day intervals during storage

Briefly, a 10 °C increase in temperature corresponded to a 6–7 °C increase in the rate of autolysis.

In calculating the degree of autolysis, the initial, nonlinear, sequence of the time-activity relation was omitted, since in that period, despite the inhibited metabolism, activity may have been released from cells into the spar-

Table 3

Degree of the autolysis of champagne yeast after 100 days of storage

Conditions of storage of champagne	Δ dpm for 100 days	Quantity of autolysed yeast (g yeast dry matter per litre)	Degree of autolysis per cent
4 °C no shaking	19 250	0.2125	12.3
4 °C shaking	21 500	0.2373	13.7
10 °C no shaking	23 370	0.2580	14.9
10 °C shaking	26 010	0.2870	16.6
20 °C no shaking	34 150	0.3770	21.8
20 °C shaking	37 510	0.4150	24.0
30 °C no shaking	43 450	0.4800	27.8
30 °C shaking	49 800	0.5500	31.7
40 °C no shaking	54 830	0.6052	35.0
40 °C shaking	61 350	0.6772	39.2

kling wine without autolysis and may have caused the initial rapid increase in activity.

DRBOGLAV and co-workers (1970) examined factors influencing yeast autolysis, using ^{14}C-alanine. They observed hardly any autolysis by the end of the first 6-day period of incubation at –5 °C or +5 °C. At 18 °C, very little autolysis ensued in the first 3–6-day period, whereas 90–96% of the yeast cells autolysed in 6 h at 40 °C. The autolysis was considerably accelerated by stirring the mixture. Stirring at 18 °C resulted in an appreciable autolysis within 3 h, whereas the autolysis was very weak without stirring, even after 6 h of incubation.

Our results are similar in character, yet numerically different, from those of DRBOGLAV and co-workers (1970). In our experiments, the autolysis was weaker in general, the difference being especially well-expressed at relatively high temperatures. We observed less pronounced enhancement in samples which were shaken at intervals than the cited authors did in continuously stirred samples.

We used *Champagne Hautvillers* yeast and tank-fermented sparkling wine. Considering that different yeasts are practically identical in composition, especially in the composition of the cell wall, and that the sparkling wine used by us is of average composition as regards alcohol content and acidity, generalization of our observation might seem to be acceptable.

It should be noted that an acceleration of autolysis by incubation at an elevated temperature may be unfavourable as regards organoleptic grading of sparkling wine because cell components dissolved in the latter are not bound

in a secondary chemical reaction, and the resulting sparkling wine may taste like yeast.

Taking into account the fact that the bottle-fermented sparkling wine reaches its best quality in the second year of seasoning, it seems to be reasonable to assume that further, secondary processes may take place after the enzyme activity has settled. The development of the classical sparkling wine character may be attributed to such processes.

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CHANGES IN THE ACTIVITIES OF CERTAIN ENZYMES OF CHAMPAGNE YEAST DURING STORAGE OF SPARKLING WINE

I. MOLNÁR, E. OURA and H. SUOMALAINEN

(Received 13 September 1979; accepted 5 January 1980)

Active and NaF-inhibited *Champagne Hautvillers* champagne yeast were added to tank-fermented sparkling wine and the activities of the following yeast enzymes were serially measured during storage at different temperatures: alcohol dehydrogenase, aldolase, esterase, glucose-6-phosphate dehydrogenase, glycerolaldehyde-3-phosphate dehydrogenase, glutamate–oxalacetate transaminase, hexokinase, β-fructosidase, malate dehydrogenase and protease. On the basis of the changes in activity, the enzymes could be divided into three groups. Alcohol dehydrogenase, aldolase and glutamate–oxalacetate transaminase lost their activities most rapidly; the activities of glycerolaldehyde-3-phosphate dehydrogenase, hexokinase, and malate dehydrogenase were still considerable after a storage of 50–90 days, depending on the temperature; the β-fructosidase activity showed hardly any change, and the proteolytic activity increased, during storage. The length of the period in which, following the second fermentation, enzyme activity is still considerable is estimated between 30 and 60 days.

In the development of the classical character of champagne, the autolysis of the champagne yeast plays an outstanding role. In the case of bottle fermentation, sparkling wine is kept at cellar temperature for one or two years, together with the yeast. During this fermentation period, the yeast cells are autolysed and their cellular components are dissolved in the sparkling wine. Thus, the chemical composition of the champagne is modified and its organoleptic properties are improved. Consequently, the autolysis of champagne yeast is a desirable, even necessary, process in order to develop the champagne character. The enzymes present in the yeast presumably contribute to this character. Nevertheless, the inactivation rate of the enzymes in the sparkling wine under the prevalent conditions (acidity, alcohol concentration), *i.e.* the question of how long these enzymes play a considerable role in the course of aging after the second fermentation, has remained unknown.

The changing activities of a few hydrolytic and oxidoreductive enzymes were followed up in wine by (AVAKJANC and co-workers, 1972) during seasoning and heat treatment of wine, especially in the presence of *Sacharomyces oviformis*. The proteolytic activities reached the highest levels after one-month storage as well as after cold treatment. The peroxidase, alcohol dehydrogenase

and glutamate dehydrogenase activities, on the other hand, significantly declined. KORMAKOVA and her colleagues (1979) examined the activities of proteolytic and oxidoreductive enzymes, *viz.* alcohol dehydrogenase, lactate dehydrogenase and malate dehydrogenase, in sparkling wine aged with yeasts intact or destroyed in structure. In the presence of yeast of degraded structure, the activity of these enzymes increased. The enzyme activity in the sparkling wine was the highest after one month, and the lowest after 12 months, of aging.

These results prompted us to follow up the activities of the enzymes to be listed below while sparkling wine was kept at 10, 20 or 30 °C. We wished to estimate the length of the period during which these enzymes still may play a considerable role in the seasoning of sparkling wine under the given conditions of storage. The following enzymes, all being present in the champagne yeast, were studied: alcohol dehydrogenase, aldolase, esterase, β-fructosidase, glucose-6-phosphate dehydrogenase, glycerolaldehyde-3-phosphate dehydrogenase, glutamate-oxalacetate transaminase, hexokinase, malate dehydrogenase, and protease.

1. Materials and methods

1.1. Microorganism

Champagne yeast *Champagne Hautvillers* maintained on slant malt agar was used throughout.

1.2. Culture medium used for the production of champagne yeast

A synthetic medium was used which contained 100 g glucose, 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 $MgSO_4 \cdot 7\ H_2O$, 4 mg $MnSO_4 \cdot H_2O$, 0.5 mg $CuSO_4$, 2.5 mg $ZnSO_4 \cdot 7\ H_2O$, 125 mg inositol, 6 mg Ca-pantothenate, 6 mg pyridoxine, 5 mg thiamine, 5 mg nicotinic acid amide and 0.2 mg biotin, each per 1 litre. The pH of the medium was adjusted to 4.5 with $2\ N\ H_2SO_4$. The medium was autoclaved at 115 °C for 30 min. The fermentation was performed in an LKB BIOTEC fermentor continuously operating in anaerobic conditions. N_2 gas presterilized by filtration was driven through the fermentor at a rate of 60 l h^{-1}, under continuous stirring. Fermentation temperature: 28 °C; pH 4.5. The rate of medium inflow was 240 $cm^3\ h^{-1}$. The liquid leaving the fermentor by overflow was collected in an ice bath and the yeast cells were sedimented by centrifuging in the cold. Then, the yeast cells were washed in distilled water of 0 °C.

1.3. Preparation of samples

A suspension containing 25 g yeast per l was prepared in sparkling wine produced by tank fermentation at 0 °C. The yeast had been propagated by fermentation. The bottles containing the yeast were crown-stoppered. Samples were stored at 10, 20 and 30 °C, resp. Enzyme activities were measured at 10-day intervals.

To follow up the inactivation of enzymes being present in NaF-inhibited yeast, we added 2 mg cm^{-3} NaF to wet yeast of 0.25 g cm^{-3}. The mixture was kept at 4 °C for 24 h and an aliquot of the mixture was made up with sparkling wine containing 2 mg cm^{-3} NaF to set the yeast concentration at 25 g wet yeast per l. The flasks containing the yeast in sparkling wine were crown-stoppered and samples from it were placed in thermostats of 4, 10, 20, 30 and 40 °C. Enzyme activities were assayed after 2, 4, 6, 9, 12, 24 and 48 h of incubation.

1.4. Preparation of yeast extract by disintegration of yeast cells

Samples from yeast suspensions pre-incubated in sparkling wine were centrifuged at 3 000 rpm for 10 min at 4 °C. The yeast sediments were washed twice in a potassium phosphate buffer of pH 7.0 at 0 °C and homogenized in 5 cm^3 of the same buffer, using 6 cm^3 of *Ballotini* glass beads of 1 mm diameter in a *Mickle* disintegrator (The Laboratory Engineering Co.) at 4 °C for 30 min. The glass beads were removed and the disintegrated yeast was centrifuged at 12 000 rpm for 20 min at 4 °C. Enzyme activities were assayed in the supernatants.

1.5. Protein assay

The protein concentration in yeast extracts was measured by the method of LOWRY and co-workers (1951).

1.6. Enzyme assays

Enzyme activities were measured in cell-free extracts of the disintegrated mass. The activities of alcohol dehydrogenase (EC.1.1.1.1.), aldolase (EC.4.1.2.13); glucose-6-phosphate dehydrogenase (EC.1.1.1.49.), glycerolaldehyde-3-phosphate dehydrogenase (EC.1.2.1.12.), glutamate-oxalacetate transaminase (EC.2.6.1.1.), hexokinase (EC.2.7.1.1.), β-fructosidase (EC.3.2.1.26.) and malatedehydrogenase were determined as described by BERGMEYER (1974). The values presented are expressed as specific activity per mg protein. The esterase activity was assayed as recommended by BIER (1955) and PARKKINEN and

co-workers (1978), using p-nitrophenyl acetate (p-NPA) as the substrate. The methanolic 0.1 *M* p-NPA solution used in the assay could be stored at +4 °C for a week, without any loss of activity. The enzyme reaction was carried out at 25 °C in 10 cm^3 of the reaction mixture containing 2 μmol p-NPA in 30 *mM* phosphate buffer of pH 7.0 and cell-free extract corresponding to 2.5 mg of yeast dry matter. After an incubation of 30 min, the reaction mixture was centrifuged at 1 000 g for 3 min and the absorption by the supernatant was immediately determined at 400 nm in a cuvette of 1 cm. Under these conditions, esterase activity is linearly related to the absorption. To standardize the reaction, 0.1–0.7 μmol cm^{-3} p-nitrophenol solutions were prepared, and of each standard solution 1.0 cm^3 was added to 9.0 cm^3 of 0.15 *M* phosphate buffer of pH 7.0. Absorption (A) was measured as above and illustrated as a function of the p-nitrophenol concentration. The equation describing the correlation is as follows:

$$\mu\text{mol p-nitrophenol} = 0.0079\,A + 1.1371$$

Esterase activity was calculated in μmols of p-nitrophenol released in 60 min. The given values are related to 1 mg yeast protein.

The proteinase activity was determined using azoalbumin as the substrate, according to TOMARELLI (1949). Azoalbumin of pH 7.5 was diluted in a 0.2 *M* phosphate buffer of pH 7.5 to obtain a solution containing 25 mg azoalbumin per cm^3. One cm^3 cell-free extract was added to an equal amount of the substrate and the mixture thus obtained was incubated at 37 °C for 1 h. The blank contained 1.0 cm^3 substrate with the same volume of the phosphate buffer. Eight cm^3 of a 50% trichloro-acetic acid solution were added after incubation. Thus, the digestion was stopped and the excess substrate was precipitated. The mixtures were filtered, and 5.0 cm^3 of a 0.5 *N* NaOH solution were added per 5.0 cm^3 filtrate. The ratio of absorption to blank absorption was calculated from values obtained at 440 nm in 1 cm cuvettes. The specific activity of standard *Streptomyces griseus* protease was measured simultaneously by the same method. One unit of this preparation liberates 1.0 μmol (181 μg) tyrosine during 1 min at 37 °C and pH 7.5. The specific absorption by the yeast protease was obtained by relating the measured absorption to the absorption by the standard. The specific activity is expressed in nmol tyrosine per min per mg protein. The data represent specific activities per mg yeast protein.

2. Results and conclusions

Figures 1–10 illustrate the changes during storage in the activities of the above-mentioned enzymes of the *Ch. Hautvillers* champagne yeast under the

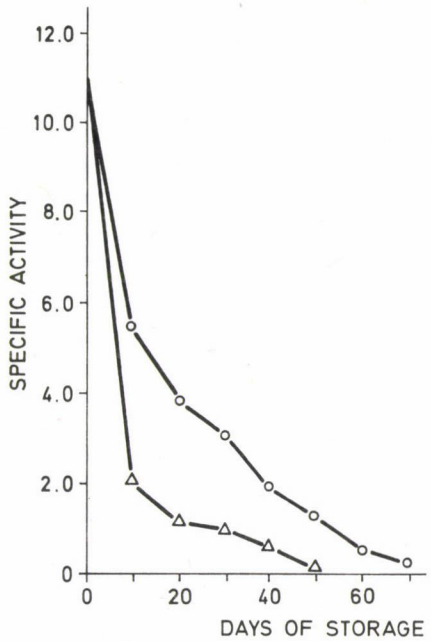

Fig. 1. Dynamics of the alcohol dehydrogenase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in U per mg protein

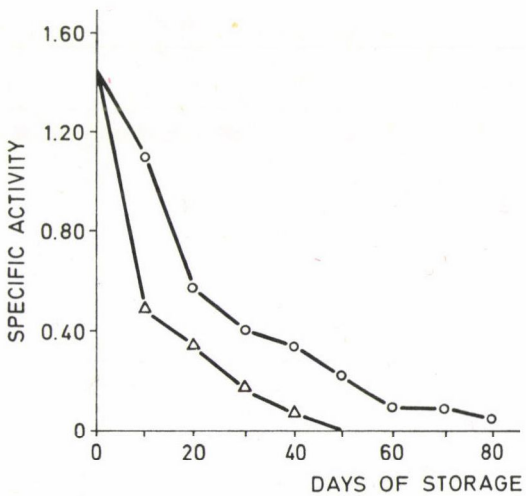

Fig. 2. Dynamics of the aldolase activity of *Ch. Hautvillers* yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in U per mg protein

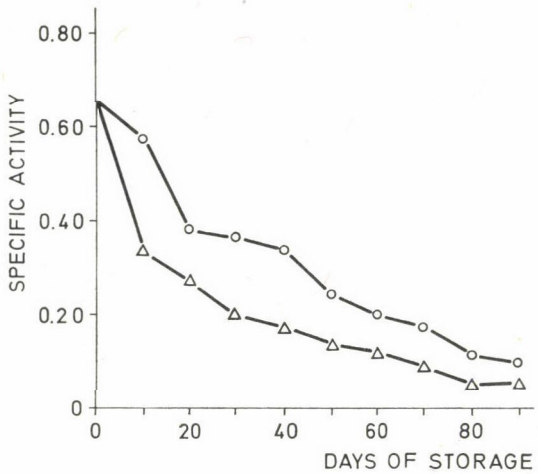

Fig. 3. Dynamics of the esterase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in μmol p-nitrophenol per min per mg protein

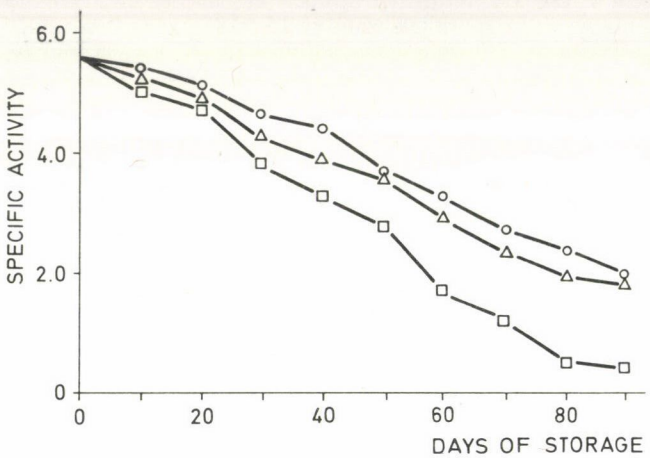

Fig. 4. Dynamics of the β-fructosidase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–), 20 °C (–△–△–) and 30 °C (–□–□–)
Specific enzyme activity is expressed in μmol

given conditions. Table 1 shows the specific activities after storage for 10, 50 and 90 days, expressed in per cent of the original activities in the champagne yeast. The same with the enzymes of NaF-inhibited champagne yeasts are shown in Table 2.

Table 1

Changes in enzyme activities of Champagne Hautvillers *champagne yeast during storage in sparkling wine*

Designation of the enzyme	Activity expressed in per cent of initial activity after					
	10-day	50-day	90-day	10-day	50-day	90-day
	storage at					
	10 °C			20 °C		
Alcohol dehydrogenase	50.2	11.7	–	19.0	1.1	–
Aldolase	77.8	15.1	3.5	23.0	1.5	–
Esterase	87.2	36.7	15.0	50.7	20.5	9.4
β-fructosidase	95.0	65.5	35.0	92.9	62.3	32.0
Glucose-6-phosphate dehydrogenase	81.6	28.8	4.5	51.1	3.4	–
Glycerolaldehyde-3-phosphate dehydrogenase	71.6	52.0	23.2	49.2	7.3	–
Glutamate-oxalacetate transaminase	54.3	9.4	–	30.5	–	–
Hexokinase	78.5	41.2	11.0	54.6	16.5	1.3
Malate dehydrogenase	86.0	69.3	56.5	54.2	21.3	–
Protease	78	299	484	273	983	1416

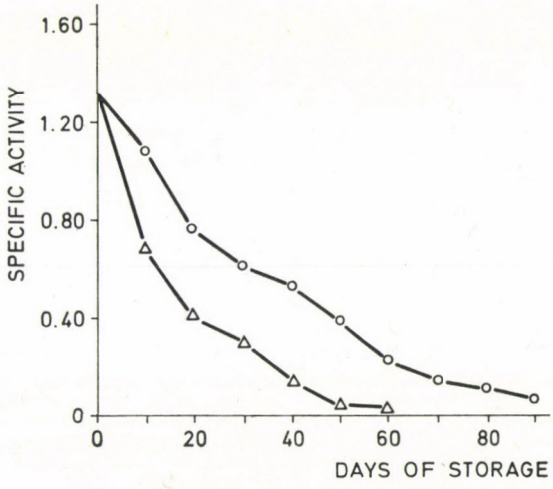

Fig. 5. Dynamics of the glucose-6-phosphate dehydrogenase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–) Specific enzyme activity is expressed in U per mg protein

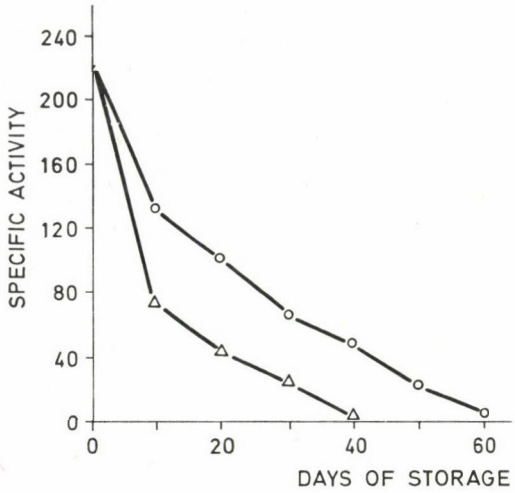

Fig. 6. Dynamics of the glutamate–oxalacetate transaminase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in mU per mg protein

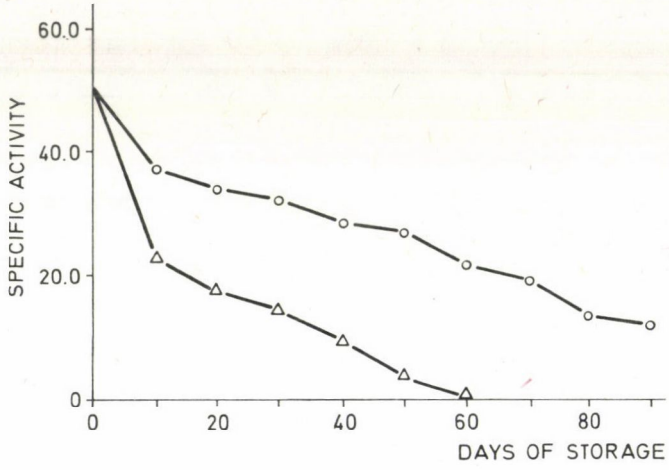

Fig. 7. Dynamics of the glyceraldehyde-3-phosphate dehydrogenase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–△–△–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in U per mg protein

The enzymes under study presumably represent the enzymes active in yeast cells under anaerobic conditions. Similarly, the average changes in demonstrable activities are thought to represent the changes in the activities of the same enzymes in the champagne yeast cells.

The questions to which we are looking for an answer are the following: (a) how do the activities of the enzymes in champagne yeast change as a function

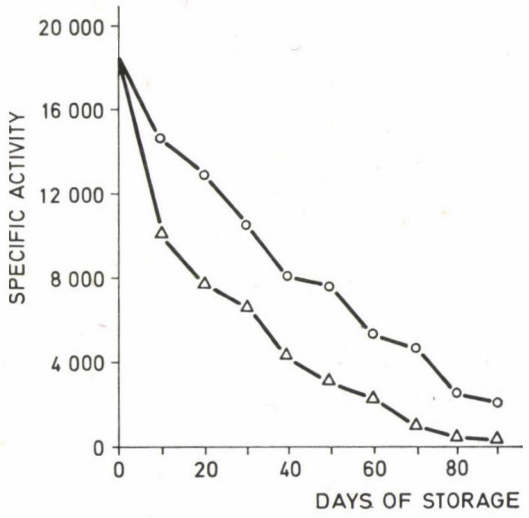

Fig. 8. Dynamics of the hexokinase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in U per mg protein

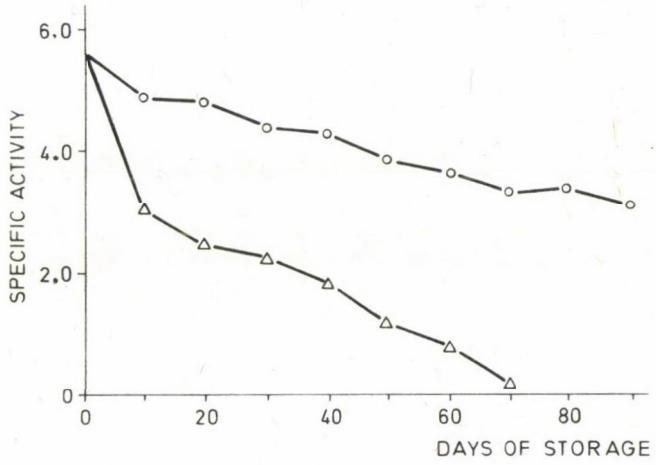

Fig. 9. Dynamics of the malate dehydrogenase activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–) and 20 °C (–△–△–)
Specific enzyme activity is expressed in U per mg protein

of time and temperature during the storage aimed at improving the quality, *i.e.* the period of the so-called aging, of sparkling wine (b) how active are the enzymes in the yeast during the aging that follows the second fermentation?

The enzymes under study can be divided into three groups according to the rate of inactivation during storage. In the course of aging, the activities of alcohol dehydrogenase, aldolase and glutamate-oxalacetate transaminase fell

Table 2

Changes in enzyme activities of Champagne Hautvillers *champagne yeast during storage in sparkling wine*

Designation of the enzyme	Activity expressed in per cent of initial activity after											
	2 h	6 h	24 h	2 h	6 h	24 h	2 h	6 h	24 h	2 h	6 h	24 h
	storage at											
	4 °C			10 °C			20 °C			30 °C		
Alcohol dehydrogenase	29.1	1.9	–	15.0	1.2	–	7.1	–	–	–	–	–
Aldolase	50.7	13.6	3.3	37.3	11.3	3.2	14.8	–	–	–	–	–
Esterase	76.0	58.2	20.8	69.9	46.1	16.5	49.1	18.3	13.9	29.8	7.4	3.1
β-fructosidase	97.5	96.1	94.6	94.6	97.5	95.3	95.6	92.1	91.0	95.1	95.5	93.4
Glucose-6-phosphate dehydrogenase	60.3	25.4	3.5	53.4	25.4	2.1	13.4	3.8	–	–	–	–
Glycerolaldehyde-3-phosphate dehydrogenase	43.0	17.2	–	30.2	11.2	–	7.9	–	–	–	–	–
Glutamate-oxalacetate transaminase	82.4	62.8	7.2	65.3	36.5	3.6	40.6	5.7	–	12.0	1.5	–
Hexokinase	90.0	80.6	48.3	96.3	71.2	37.4	71.7	23.4	1.9	47.3	2.6	–
Malate dehydrogenase	89.2	69.4	5.2	81.6	42.3	2.8	41.2	4.7	–	–	–	–
Protease	79.2	78.5	75.5	85.1	83.7	78.5	90.3	90.3	83.7	66.5	65.9	63.7

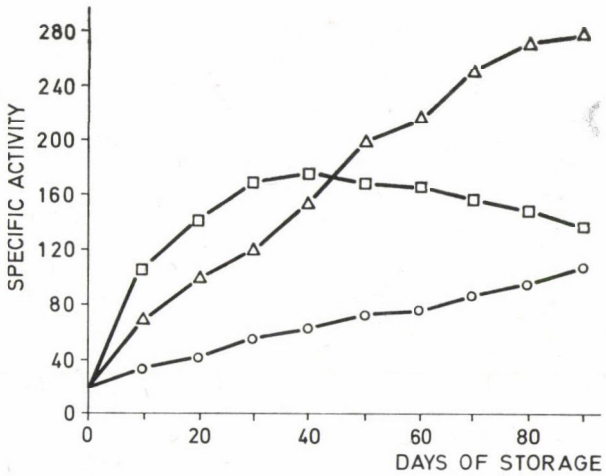

Fig. 10. Dynamics of the protease activity of *Ch. Hautvillers* champagne yeast during storage in sparkling wine at 10 °C (–○–○–), 20 °C (–△–△–) and 30 °C (–□–□–). Specific enzyme activity is expressed in μmol tyrosine per min per mg protein

at the highest rate, *viz.* to a non-measurable level in 40–50 days and 60–70 days at 20 °C and 10 °C, resp. The activities of the same enzymes inhibited by NaF ceased within a 2–6 h period of storage.

The activities of the enzymes glycerolaldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, and malate dehydrogenase were still measurable in the champagne after a storage of 3 months at 10 °C; the activities of these enzymes ceased after a 70- to 80-day storage at 20 °C. Enzyme activity of the NaF-inhibited champagne yeast in general ceased after a storage of 6 to 48 h, but at 30 °C these enzymes already lost their activities in 2–4 days.

The activities of the enzymes esterase and β-fructosidase of the champagne yeast declined slowly. These enzymes displayed considerable activities even after 3-month storage at 10 °C or 20 °C. Even at 30 °C, one-tenth of the β-fructosidase activity had remained measurable. The NaF-inhibited esterase reached the 0 level between 6 and 48 h, depending on the temperature, whereas the inhibited β-fructosidase activity remained practically unchanged, independent of the temperature.

The relative stability of the β-fructosidase is not surprising if the lack of substrate in the sparkling wine is considered. In the course of the 3-month storage, the invertase enzyme activity is presumably inactivated *via* a synthesizing reaction, not a decomposing one (AVAKJANC, 1965; AVAKJANC & BELOUSOVA, 1965; AVAKJANC & GULJAEVA, 1968).

The proteolytic activity of the champagne yeast was continuously increasing because of incativation of the protease inhibitor during storage (TAKEYORI *et al.*, 1975).

In the NaF-inhibited yeast, the enzymes needed 2 to 48 h to be inactivated, depending on the temperature. All the enzymes that had found their substrate(s) in the yeast or in the sparkling wine lost their activities rapidly. In the lack of any substrate of β-fructosidase, the activity of this enzyme did not change, and the proteolytic activity remained at a low level, due to the inhibitory effect. Taking into account the rapid inactivation of the enzymes under study and the fact that we always measured activity deriving from living cells, the long-lasting activity measured in the experiments may be explained by a numerical decrease in living cells.

Anyhow, the enzymes of the champagne yeast added to sparkling wine had lost a considerable part of their activities by the 20th day of storage. On days 80–100, there was practically no activity demonstrable in the yeast, *i.e.* a further development of the sparkling wine could not be influenced by residual yeast enzyme activities.

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QUANTITATIVE DETERMINATION OF MUSCLE PROTEIN IN FOODS CONTAINING PLANT OR OTHER PROTEINS

PRELIMINARY REPORT

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Quantitative determination of muscle protein in the presence of other proteins is possible by the red colour reaction it gives in alkaline medium with creatine, diacetyl and alfa-naphtol. The intensity of the colour formed is proportional to the creatine content. By means of a calibration line, the meat protein content of the sample can be calculated from the optical density values measured by spectrophotometry. In the case of heat-treated products the result is not affected by the kind of meat in the product (pork or beef). The method is exact and comparatively rapid. Extraneous proteins (plant protein, caseinate, *etc.*) do not give this reaction, therefore the result is not influenced by them.

The trend of increasing the nutritionally important protein content by incorporating plant proteins is spreading in the meat processing industries all over the world. Plant protein is cheaper than muscle protein, however, of lower nutritional value because of its amino acid composition. Taking into account nutritional and economic points of view, an optimum proportion may be established for each product.

It is of fundamental importance to have a method sufficiently rapid and objective to determine quantitatively muscle protein in foods in the presence of proteins of varied origin. Acrylamide gel electrophoretic identification, chromatographic separation, histochemical microscopy and serological tests for the identification of protein of animal and plant origin are rather lengthy procedures, not too suitable for systematic routine analysis (KIERMEIER, 1968; VÁLAS-GELLEI, 1977). A quantitative determination method seems to satisfy the above requirements (KHAN & COWEN, 1977). In this method, the determination of protein of animal origin is based on the measurement of creatine.

Creatine is a characteristic component of animal muscle tissue containing nitrogen (*N*-methyl-guanidinoacetic acid) participating in the metabolism of phosphorus. As an effect of enzymatic processes or in the course of industrial processing, heat treatment may convert creatine into derivatives, such as for instance creatinine. The quantity of creatine varies with various parts of the body; according to KHAN and COWEN (1977), beef muscle contains 3.5–5.1 mg g^{-1}. According to other data in the literature (DAHL, 1963), the joint

creatine and creatinine content of pork or beef amounts to 2.42–2.56% related to the total protein content.

Creatine may be determined by the *Voges-Proskauer* reaction as modified by *Baritt*. The essence of this method is that creatine reacts in an alkaline medium with alfa-naphtol and diacetyl to form a red compound the colour intensity of which is proportional to the creatine content (WONG, 1971).

A calibration line may be constructed from the photometrically established creatine content of samples containing a known amount of protein of animal and plant origin and by plotting the optical density values (according to the above method) *vs.* total *N* content. The muscle protein content may be calculated from the creatine content determined as described above by means of the calibration line (KHAN & COWEN, 1977). Several authors investigated the conditions of the reaction, the reproducibility and accuracy of the method (EGGLETON *et al.*, 1943).

In the course of the present study, the data obtained by KHAN and COWEN for beef were extended over pork, the mixture of pork and beef and the results obtained for raw meat and heat-treated meat were compared.

1. Materials and methods

1.1. Reagents

Trichloroacetic acid (TCA): 10 g dissolved in 100 cm^3 water
Sodium hydroxide-sodium carbonate buffer: 60 g NaOH + 160 g Na_2CO_3 dissolved in 1 000 cm^3 water
Alfa-naphtol buffer, 1 g dissolved in 100 cm^3 water (freshly prepared)
Diacetyl, 0.1 g in 100 cm^3 water (freshly made)
Sulfuric acid, concentrated
Wet ashing reagent (K_2SO_4, Se, $CuSO_4$)
HCl, 0.1 *N*
NaOH, 0.1 *N*
NaOH, 33 g dissolved in 100 cm^3 water
Quartz sand

1.2. Materials

Leg of beef
Pork ham
Soy concentrate GL-750 70% (Central Soya Co., USA)
Na caseinate, EM-HV (Zuid-Nederlandsche Melkindustrie N. V., The Netherlands).

1.3. Measurement of creatine

A sample of 10.00 g was homogenized with 50.00 cm^3 of TCA, then centrifuged for 20 min at 3 000 rpm. The liquid phase was transferred to a 200 cm^3 volumetric flask. The solid residue was twice rinsed with TCA, and centrifuged. The solutions were united and filled up to 200 cm^3. After allowing to stand for 2 h at 20 °C, the solution was filtered. To 1 cm^3 of the filtrate 5.00 cm^3 buffer and 3.00 cm^3 of alfa-naphtol solution and 2.00 cm^3 of diacetyl solution were added and the mixture was filled up to 25 cm^3. In the case of heat-treated samples 3.00 cm^3 of the filtrate were used.

The compensating solution for photometry was of the same composition except for the filtrate instead of which TCA was added. Measurement was carried out in a cuvette of 1 cm^3 after allowing to stand for 20 min at room temperature, at 520 nm. A PYE UNICAM SP8-100 spectrophotometer was used.

1.4. Total nitrogen content

The *Kjeldahl* method was applied according to the HUNGARIAN STANDARD (1954).

1.5. Determination of the solids content

To obtain the solids content, needed in further calculations, the HUNGARIAN STANDARD (1953) was used.

1.6. Preparation of the test sample

The meat was minced twice. The granulated soya concentrate was soaked in water twice its weight. Sample units of 50 g were made of the minced meat and soaked soya by weighing on the precision balance 0, 10, 20, 30, 40 and 50 g from one material and complementing it with the other. After homogenization the samples were stored in a refrigerator at 4 °C in airtight packing. These were the raw samples. The same mixtures packaged in cans were heat treated at 115 °C, applying 10 min to reach the temperature, keeping it for 45 min and cooling in 10 min (heat-treated samples).

2. Results

The calibration curve was plotted on the basis of the following sample series:

- beef-granulated soya (raw, heat treated; Tables 1 and 4)
- pork-soya granulate (raw, heat treated; Tables 2 and 5)

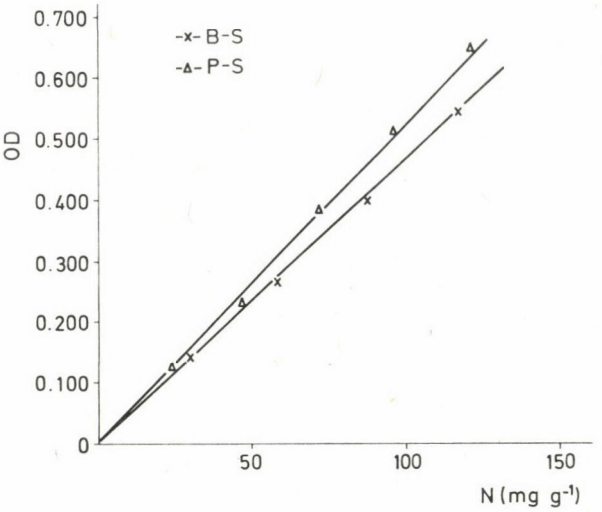

Fig. 1. Correlation between muscle nitrogen (mg g^{-1}) and optical density (OD) in the creatine reaction in raw mixtures of beef + soya (B-S) and pork + soya (P-S)

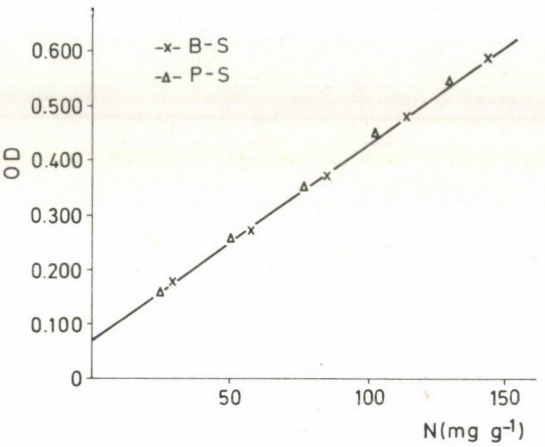

Fig. 2. Correlation between muscle nitrogen (mg g^{-1}) and optical density (OD) in the creatine reaction in heat-treated mixtures of beef + soya (B-S) and pork + soya (P-S)

– pork-beef (raw, heat treated; Tables 3 and 6).

The results are illustrated in Figs. 1—2.

3. Conclusions

Linear correlation was found between muscle protein and optical density proportional to the creatine content in all the samples whether raw or heat-treated. The difference between the slope of the lines belonging to raw pork and beef, resp., was minimal.

Table 1

Analytical data of raw beef–soya mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
B	S				mean value	standard deviation
100	0	24.92	147.28	147.28	0.699	0.019
80	20	26.20	139.05	117.84	0.543	0.011
60	40	27.60	128.68	88.38	0.402	0.024
40	60	28.90	122.69	58.92	0.270	0.024
20	80	29.50	115.39	29.46	0.146	0.015
0	100	31.00	107.92	0.00	0.004	0.016

B = beef
S = granulated soya–water mixture (1 : 2)

Table 2

Analytical data of raw pork–soya mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
P	S				mean value	standard deviation
100	0	26.18	122.15	122.15	0.647	0.015
80	20	27.02	117.47	97.72	0.507	0.019
60	40	28.50	109.42	73.29	0.385	0.024
40	60	29.00	109.00	48.86	0.235	0.005
20	80	29.28	108.70	24.43	0.127	0.009
0	100	30.82	103.20	0.00	0.004	0.010

P = pork
S = granulated soya–water mixture

In the case of the heat-treated samples all the points of measurement fall practically on the same line. This means that the creatine content, in close correlation with muscle protein, is independent of the origin of the meat. To obtain optical density values suitable for evaluation, 3 cm^3 samples of the heat-treated meat had to be subjected to photometry. Since the arginine content of soya protein is relatively high, it is possible that, as an effect of heat treatment, the guanidino group of this compound reacts with the reagent.

Table 3

Analytical data of raw beef–pork mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
B	P				mean value	standard deviation
100	0	26.54	127.79	127.79	0.657	0.015
80	20	25.96	130.76	131.26	0.675	0.025
60	40	26.02	134.32	134.74	0.681	0.023
40	60	26.10	137.82	138.22	0.692	0.013
20	80	26.04	141.48	141.70	0.716	0.025
0	100	25.15	145.16	145.16	0.724	0.012

B = beef
P = pork

Table 4

Analytical data of heat-treated beef–soya mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
B	S				mean value	standard deviation
100	0	24.44	146.44	146.44	0.598	0.015
80	20	25.78	131.57	117.16	0.480	0.023
60	40	27.19	129.25	87.87	0.372	0.025
40	60	28.59	120.53	58.58	0.271	0.022
20	80	29.95	114.59	29.29	0.176	0.014
0	100	31.28	106.50	0.00	0.075	0.026

B = beef
S = granulated soya–water mixture

This may be the explanation for the phenomenon that the optical density of the sample containing only soya increased upon heat treatment. The lower optical density values obtained in heat-treated samples may be due to the change in creatine caused by heat treatment.

Na-caseinate is frequently used as an additive in meat products, therefore its effect upon the muscle protein analysis was investigated. Sodium caseinate does not give colour reaction with alfa-naphtol and diacetyl reagents, thus its presence does not influence the result.

Table 5

Analytical data of heat-treated pork–soya mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
P	S				mean value	standard deviation
100	0	26.02	130.66	130.66	0.558	0.015
80	20	26.98	124.46	104.52	0.453	0.025
60	40	28.07	116.64	78.39	0.359	0.024
40	60	29.90	112.13	52.26	0.255	0.012
20	80	30.28	108.72	26.13	0.158	0.023
0	100	31.30	106.42	0.00	0.061	0.023

P = pork
S = granulated soya–water mixture

Table 6

Analytical data of heat-treated beef–pork mixtures

Composition of sample (%)		Solids content (%)	Nitrogen in the solids (mg g^{-1})	Muscle protein nitrogen in the solids (calculated) (mg g^{-1})	Optical density	
B	P				mean value	standard deviation
100	0	24.44	146.44	146.44	0.601	0.013
80	20	24.65	142.33	142.87	0.593	0.014
60	40	25.00	139.76	139.31	0.586	0.022
40	60	25.31	135.90	135.74	0.579	0.022
20	80	25.65	131.04	132.18	0.566	0.012
0	100	26.02	128.62	128.62	0.556	0.013

B = beef
P = pork

The effect of gelatine, tendon and rind was also tested. They were found to give a slight reaction with alfa-naphtol-diacetyl reagents. The optical density value for gelatine and tendon tissue was about one fiftieth of that for beef, while the value for rind was about one twentieth.

The accuracy of photometric measurement was checked by mathematical calculations. The equations for the calibration curves, constructed from the data were calculated (SVÁB, 1973) and these proved the correctness of the method and the conclusions (Table 7).

Table 7

Mathematical evaluation of the samples

Composition of sample	Equation of regression line	Correlation coefficient
Raw:		
beef–soya	$Y = 0.018–0.017\ X$	$r = 0.94$
pork–soya	$Y = 0.020–0.013\ X$	$r = 0.99$
beef–pork	$Y = 0.024–0.018\ X$	$r = 0.94$
Heat treated:		
beef–soya	$Y = 0.024–0.063\ X$	$r = 0.98$
pork–soya	$Y = 0.022–0.064\ X$	$r = 0.99$
beef–pork	$Y = 0.025–0.068\ X$	$r = 0.91$

Y = optical density value
X = muscle nitrogen (mg g^{-1})

It was established by the F test that the difference between regression equations, relating optical density (Y) to muscle protein (X), was not significant. Thus a common regression equation was found:

$$Y = 0.021 - 0.015\ X.$$

The conclusions of the study being favourable, it seems desirable to determine the muscle protein content of canned meat products (meat-hash, lunchon meat) in order to prove the practical applicability of the method.

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ANTHOCYANIN PIGMENTS OF THE BLACK CHERRY

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Pigment was prepared in solid state for analysis from the short-stemmed black cherry variety *Szomolya*. The anthocyanins were extracted from the aqueous solution by *Dowex* 50 WX 4 resin, and after eluting with methanol they were precipitated with diethyl ether. The pigments were separated by paper chromatography on *Whatman* No. 3. paper into four fractions with a solution of acetic acid-cc. hydrochloric acid-water (15 : 3 : 82). The first and fourth spot contained 7% of the material. The two major components – amounting to 93% of the pigment – were identified; the second spot from the start point was cyanidin-3-(p-coumaroyl-)-glucoside, the third spot was cyanidin-3-(p-coumaroyl)-rhamnoglucoside; the ratio of the two compounds was 1 : 3.

Colour is a value-measuring characteristic of fruits and fruit products, which can be altered during the processes of preservation. Sometimes it occurs, that the raw material does not contain sufficient pigment. In these cases synthetic food colours are used according to need. Due to sanitary considerations, there is a world trend to suppress the use of artificial food colours. Stress is laid upon the use of natural colours and on the development of technologies which preserve the natural colour. According to GOODWIN (1976) the stability of the pigments depends partly on the chemical structure, and, therefore, the identification and the determination of the chemical structure of the pigments of the processed fruits is important. It is possible to draw conclusions from them on their stability. The present paper reports on the pigments of the short-stemmed black cherry (variety *Szomolya*), which is a black-coloured popular export fruit indigeneous to the neighbourhood of Eger.

1. Materials and methods

1.1. Preparation of the pigments

The cherries were refrigerated in the refrigerating plant of the cooperative farm *Szőlőskert* at Nagyréde and they were put into circulation in 1979.

Three hundred and fifty g of the fruit were reduced to pulp and the pigments were extracted with methanol containing 1% (v/v) HCl. The alcoholic solution was diluted tenfold with water and the pigments were bound on *Dowex* 50 WX 4 resin. After elution with 1 volume of warm methanol containing 1 (v/v) HCl the greatest part of the alcohol was distilled off *in vacuo* at 40 °C. From the remaining concentrated solution the pigments were precipitated with diethyl ether, the precipitate was filtered, washed and dried. This material was used for analysis and identification.

1.2. Separation of the pigments

Very good separation was obtained on *Whatman* No. 3. paper with acetic acid–cc. hydrochloric acid–water (15 : 3 : 82) solution. About 2 mg of the pigment – dissolved in methanol – was deposited on a 20×50 cm sheet in the form of a band. After developing for 15 h the pigments were eluted from the separated bands with methanol by descending technics. The solutions were evaporated to dryness and the analysis was performed.

1.3. Identification of the acylating acids

The acids linked to the sugar components of the pigments were identified by the method of BIRKOFER and co-workers (1965). About 5 mg of the individual fractions were treated in a current of nitrogen with 5 cm^3 of 2% NaOH solution during 3 h at room temperature. Then the pH was adjusted to about 6 with IRC 50 resin. The resin was filtered and washed with water and methanol. The alcohol was removed from the filtrate by vacuum distillation and the acylating acids were extracted from the remaining aqueous solution with diethyl ether. The acids were identified from this solution by descending paper chromatography on *Whatman* No. 1 paper. The solvent used was the upper phase of the n-butanol–glacial acetic acid–water (4 : 1 : 5) system. Control substances were ferulic acid, p-coumaric acid and caffeic acid. Detection was performed with a freshly prepared solution of diazotized p-nitroaniline.

1.4. Determination of the glycosidic structure

One to two mg of the deacylized remainder of the individual bands were partially hydrolysed according to ABE and HAYASHI (1956). Samples were taken at different intervals and investigated by chromatography on *Schleicher & Schüll* 2043 b paper. The solvent used was the upper phase of the n-butanol–cc. hydrochloric acid–water (7 : 2 : 5) system. The dried chromatogram was developed for the second time – deviating from the procedure described in the

literature–with a solution of acetic acid–cc. hydrochloric acid–water (15 : 3 : 82). By this method, even overlapping spots were separated.

1.5. Identification of the aglycons

Two mg of the pigments were hydrolysed with 2 *N* hydrochloric acid on a boiling water bath for 30 minutes according to HRAZDINA (1970). The aglycons were separated from the sugars by binding them on a 3×1 cm column filled with *Polyclar* AT. The aglycons were eluted with methanol and the solution was evaporated *in vacuo*. The dry remainder was used for the identification. In the course of this, R_f values were determined, colour reaction was produced with aluminium chloride, the absorption maximum was determined and the shift of the maximum caused by aluminium chloride was investigated. Then degradation with barium hydroxide was performed according to SMITH and LUH (1965) and the hydroxy acids referring to the structure were identified by chromatography. Control substances were protocatechuic acid, vanillic acid, gallic acid, syringic acid and phloroglucinol. The latter is formed during the decomposition of all anthocyanidins. Diazotized p-nitroaniline was used for the detection.

1.6. Identification of the sugar components

After having bound the aglycons, the sugars liberated by complete hydrolysis remained in an aqueous hydrochloric acid solution. Hydrochloric acid was removed from the solution with di-n-octyl-methylamine according to RIBÉREAU-GAYON (1959) and the solution was evaporated. The sugars were identified by the chromatographic method of PARTRIDGE (1949). Sugars attached to C_3 of the aglycons were identified by the method of CHANDLER and HARPER (1961). Four mg of the original or of the deacylized pigment were dissolved in 0.8 cm^3 of methanol and 0.15 cm^3 of 30 per cent hydrogen peroxide was added. After 4 h the excess of the hydrogen peroxide was decomposed with *Pd* catalyst, and after 20 hours the sugars attached to the C_3 were separated from the aglycon with cc. ammonium hydroxide. This was followed by the identification by the method described above.

1.7. Determination of the proportion of the pigments

The chromatogram of the pigments was investigated with ERI 10 (Carl Zeiss, Jena) instrument and the proportion of the components in per cent was determined according to GOMBKÖTŐ (1964).

Fig. 1. Chromatogram of the pigments of the short-stemmed black cherry *Szomolya* on *Whatman* No. 3 paper. Solvent: acetic acid—cc. hydrochloric acid—water (15 : 3 : 82). Ascending technics

2. Results

From 350 g of pulp of the short-stemmed black cherry variety *Szomolya*, the anthocyanins were extracted and bound on a cation-exchange resin. After elution the pigments were precipitated with diethyl ether. This procedure yielded about 1 g of solid product, which was separated by paper chromatography into four components. The chromatogram of the pigments is shown in Fig. 1.

The proportion of the components was also determined. Bands 1 and 4 together contained about 7%, Band 2 about 23%, Band 3 70% of the pigment.

The two latter components are considered as the main pigment of the black cherry. Data of analysis are shown in Tables 1 to 3.

Table 1

$R_f \times 100$ values of the anthocyanins in different solvents

Number of the band	Solvents			
	A	B	C	D
1	7	–	–	–
2	31	47	8	28
3	47	38	12	23
4	52	–	–	–

A: acetic acid–cc. hydrochloric acid–water (15 : 3 : 82)
B: n-butanol–glacial acetic acid–water (4 : 1 : 5), upper phase
C: 1 per cent hydrochloric acid
D: n-butanol–2 *N* hydrochloric acid (1 : 1), upper phase

Table 2

Identification data of the aglycons

Number of the band	$R_f \times 100$ in Forestal's solvent	Absorption maximum (nm)	Shift of the maximum caused by $AlCl_3$ (nm)	Acid obtained by degradation with $Ba(OH)_2$
2	51	540	+38	Protocatechuic acid
3	51	540	+38	Protocatechuic acid
cyanidin	51	540	+38	Protocatechuic acid

Forestal's solvent: glacial acetic acid—cc. hydrochloric acid—water (30 : 3 : 10)

Table 3

Results of the investigation of the glycosidic structure

Number of the band	Structure according to partial hydrolysis	Sugars obtained by degradation with H_2O_2	Sugars obtained by total hydrolysis	Acylating acid
2	3-monoside	glucose	glucose	p-coumaric acid
3	3-oligoside	rutinose	glucose, rhamnose	p-coumaric acid

3. Conclusions

On the basis of the analytical data in Tables 1 to 3 it was established that of the two main pigments of the investigated black cherry – on the chromatogram developed by the given solvent – Band 2 is cyanidin-3-(coumaroyl)-glucoside, Band 3 cyanidin-3-(p-coumaroyl)-rhamnoglucoside, in the ratio 1 : 3. In addition to the elucidation of the chemical structure of the pigments methodological experiences obtained during the analysis are also given. The author believes that the solvent acetic acid–cc. hydrochloric acid–water (15 : 3 : 82) is useful for separation of larger amounts of materials for analytical purposes. It is easy to handle and has good separating qualities. It may be used advantageously where the number of the sugars bound to the aglycons is different.

In contrast to earlier practice, it was established that during the verification of the glycosidic structure it is suitable to develop the chromatogram of the partially hydrolysed product successively with different solvents. In this way, even overlapping spots can be reliably separated from each other. This is very important because it is possible to draw conclusions on the structure from the number of the spots.

*

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THE INFLUENCE OF IRON CONTAMINATION ON THE SENSORY PROPERTIES OF LIQUID FOODS

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The sensory quality of foods is highly affected by metal ions. The aim of this study was to detect the effect of iron ions, corrosion products of carbon steel construction materials, on the sensory properties of liquid foods. In comparison to the changes brought about by corrosion products in the sensory properties of liquid foods, those of ferrous and ferric salts and their anions were also investigated.

On the basis of sensory tests, the liquid foods used in the study were divided into three groups: the first group is formed by fruit juices in which a change of taste can be observed at a ferrous ion concentration of 1000 mg dm^{-3}; in the second group consisting of wines and liqueur, the threshold value was found to be at 100 mg dm^{-3}; the third group proved to be most sensitive to contamination by ferrous ions with a threshold value of 10 mg dm^{-3}. Milk and beer were assigned to the third group.

It was also established that the threshold value was highly influenced by the method used for sensory evaluation.

The investigations were not extended over the effect of iron ions on changes occurring during storage of the foods.

In the course of processing, foods may get into contact with metal construction materials. Under these circumstances the extent of corrosion is determined by the corrosion resistance of the construction material and the corrosive effect of the food and thus more or less metal ions get into the food. For certain foods, the tolerated iron ion concentration is specified by standards (*e.g.* products of the canning industry, wine). For most foods, however, such standard specifications have not been established.

Earlier examinations were carried out to establish the extent of corrosion of aluminium and steel construction materials in various kinds of liquid foods and to develop a method for detecting the dissolved metal ions (BÖRÖCZ-SZABÓ, 1976; BÖRÖCZ-SZABÓ, 1977).

The present study is aimed at establishing the effect of corrosion products of carbon steel construction materials on the sensory properties of liquid foods.

In addition to corrosion products, the effect of ferrous and ferric salts and the anions of these salts on the sensory properties of liquid foods, was also investigated for the sake of comparison.

Although iron is an important body-building element, a component of the red blood corpuscles, its presence in foodstuffs above a certain level is undesirable because they cause or catalyze changes, mainly during storage.

The greatest change brought about by metal ions was observed in the colour of foodstuffs therefore this problem formed the subject of many studies (SZÉCHÉNYI & GELLÉRT, 1958; MÜLLER, 1960; TÖRLEY, 1960; SZÉCHÉNYI, 1964). Other sensory properties were not investigated to such depth. Some information, related to orange juice, maintained that as little as 5 ppm iron or copper caused off-flavour (SHRADER & JOHNSON, 1934). DAVIES (1936) found that iron present in milk in the form of lactate lent it a metallic flavour already at a concentration of 15 ppm, CASH (1975) gave a detailed review on the effect of metal ions upon food and tried to demonstrate the intricate nature of this question. The data to which she refers were mainly published around 1930 and are not the results of her own investigations. MUNETA (1975) sums up the influence of metal ions on foods in the following:

- They are needed to a certain concentration, but above this they may be unwholesome.
- They affect the colour by forming complexes with the pigments. Iron ions are most detrimental in this respect.
- Copper and iron ions take a major part in catalyzing rancidity and degradation of taste.
- Copper and iron ions catalyze breaking down of vitamins A, C, E and K and of unsaturated essential fatty acids.

He stresses, however, that trace amounts of iron and copper are necessary for the sound functioning and growth of the human organism. In certain groups of the population, iron deficiency can be detected and it was thought to defeat this by enriching food with iron. Iron deficiency is most frequent in women and newborn babies and is closely related to their iron metabolism. Ferrous sulfate is the compound most advantageously utilized by the human organism. In comparison, other iron compounds are utilized to a degree between 0 and 100% (SPIVEY-FOX, 1971).

It is not indifferent, however, how the sensory quality of a food is affected by the naturally present, unintentionally introduced or added iron, and how the hedonic value of the food is affected by efforts to improve the sound functioning of the body.

1. Materials and methods

1.1. Materials

1.1.1. Liquid foods used in the experiments. Since the most important component of most foodstuffs is their moisture content, water distilled in laboratory glassware as well as tap water corresponding to the requirements of the HUNGARIAN STANDARD, (1978) were tested.

Fruit beverages were prepared from sour-cherry stock juice and apple juice concentrate.

The sour-cherry stock juice, product of the *Nagykőrös Canning Factory*, contained a large percentage of fibrous sediment. The juice used in the experiments was decanted from the sediment. The beverage was prepared from 300 cm^3 stock juice, 102 g sugar and 2.2 g citric acid and made up to 1 000 cm^3. Since the dissolved solids content of the sour-cherry juice was high, it had to be further diluted in order to get a refractive index corresponding to 15% soluble solids ($R_s = 15\%$). Every sour-cherry beverage used in the experiment was adjusted exactly to $R_s = 15\%$.

The apple beverage was prepared from apple juice concentrate, manufactured in the *Budapest Canning Factory*. The concentrate was diluted with fresh tap water and 2 g dm^{-3} citric acid was added. The R_s of the beverage was set between 10 and 12%.

The pasteurized milk, used in the experiments, was a commercial product (HUNGARIAN STANDARD, 1973) packaged in plastic bags.

The beer used in the experiments was a commercial product, manufactured according to HUNGARIAN STANDARD (1975) under the trade name *Kinizsi*.

Two kinds of wine were used: a red wine *Extra rubin* and a table wine *Nagykátai fehér* (White of Nagykáta), both obtained from the *Budafok Winery*.

A commercial *Császárkörte likőr* (White butter-pear liqueur) obtained from the store of the *National Enterprise of the Alcohol Industry*, was used as a liqueur.

1.1.2. Iron salts used. The ferrous salts, $FeCl_2 \cdot 4H_2O$ and $FeSO_4 \cdot 7H_2O$, and the ferric salts, $FeCl_3 \cdot 6H_2O$ and $Fe_2(SO_4)_3$, were applied in the experiments. The iron salts were of high purity analytical grade.

1.1.3. Test samples used in establishing the effect of corrosion products. The carbon steel plates used were of S3F quality. (HUNGARIAN STANDARD, 1971) The steel plate was cut into 50×100 mm test samples, which were polished to metal purity. Naturally, carbon steel must not get in direct contact with food, however, it proved to be a suitable model in these experiments.

1.2. Methods

1.2.1. Preparation of liquid foods containing iron contamination. The iron salts were dissolved in distilled or tap water and the solutions were immediately subjected to sensory evaluation. The effect of the corrosion products was tested by keeping 5 test plates, as described in section 1.1.3 polished and defatted, in distilled or tap water at room temperature for a predetermined period. The iron-containing solution thus obtained was considered the stock solution. After determining the iron content this stock solution was used to prepare the water samples for sensory evaluations.

The fruit juices were used in the form of beverages as described in section 1.1.1. For sensory evaluation, samples were prepared with ferrous and ferric salts, with the corrosion products and a control sample was also made. The ferrous and ferric salts were added to contain a round amount of Fe^{2+}, or Fe^{3+} ions, (50, 100, 200 mg dm^{-3} *etc.*) resp. The iron salts were dissolved directly in the diluted fruit juices and subjected to sensory test within a short time. To prepare the samples containing corrosion products, 5 test plates according to section 1.1.3 were placed in the sour-cherry stock juice and in the apple juice concentrate, resp. After a predetermined storage time at room temperature, the iron content of the sour-cherry stock juice and apple juice concentrate was determined and they were diluted with the respective control juices.

The iron content of the sour-cherry juice was determined by atomic absorption spectrometry on a PERKIN-ELMER 290/B apparatus. In the case of the apple juice concentrate the standard method as used in the canning industry, was applied (SPANYÁR & KEVEI, 1961).

The pasteurized milk was freshly bought on the day of the test. The amount needed was mixed and the calculated amount of iron salt was dissolved in the calculated amount of milk. Immediately after dissolution of the salts, the samples were subjected to sensory testing. The effect of the corrosion products was tested by placing the carbon steel test plates in milk and keeping them for 24 h at 273 – 276 K. A control sample was kept under the same conditions. After removal of the test plates, the samples were subjected to sensory testing and simultaneously their iron content was determined.

The bottled beer, similarly to the milk, was opened on the day of test. Since, in the course of dissolving the iron salts in the beer, a substantial proportion of the carbon dioxide disappeared, the carbon dioxide was removed from the control sample, too. Of course, the taste of the beer was strongly affected by the removal of carbon dioxide. Within 45 min so much iron was dissolved in the beer from the carbon steel plates that the plates were kept only for a few minutes in the beer prior to subjecting it to sensory evaluation.

The wine samples were also evaluated immediately after dissolving the iron salts. Contamination of wine by corrosion products was carried out by keeping the steel plates in the wine. After determining the iron content, the desired iron content was set by diluting the samples with the control wine.

The liqueurs were tested similarly to the wines.

1.2.2. Sensory tests and their evaluation. The sensory tests were carried out according to standard DIN 10 951 by the triangular test. In most cases 10, but at least 7 panel members participated in the evaluation. In order to neutralize the effect of individual samples, slightly sweet biscuits and salted sticks were offered in between.

The results of the sensory tests were evaluated according to the table given in standard DIN 10 951.

2. Results

2.1. The effect of iron on the sensory properties of distilled and tap water

The sensory testing of distilled water was difficult, because the taste of distilled water in itself is unpleasant and it is hard to distinguish between two unpleasant tastes.

Ferrous salts dissolve in water to a relatively high concentration without causing discolouration, thus the solutions could be prepared without difficulty.

The ferric salts produced a red precipitate upon dissolution. At low concentrations the solution turned opalescent, thus it was easy to recognize the iron-containing solutions. The ferric salt solution containing Cl^- ions in distilled water was completely colourless to a concentration of 1 mg dm^{-3}, while the ferric salt solution containing SO_4^{2-} anions was colourless to a concentration of 2 mg dm^{-3} and could not be distinguished from the pure distilled water by the naked eye. Results of the sensory tests are given in Table 1.

Table 1

The effect of various iron salts and of iron contamination, present in the form of corrosion products, on the sensory properties of distilled water

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	200	10	9	×××	8	×××
$FeCl_2 \cdot 4H_2O$	100	10	5	–	5	×××
$FeSO_4 \cdot 7H_2O$	100	10	8	××	4	–
$FeSO_4 \cdot 7H_2O$	50	10	4	–	1	–
$FeCl_3 \cdot 6H_2O$	1	10	10	×××	7	×
$FeCl_3 \cdot 6H_2O$	0.5	10	3	–	2	–
$Fe_2(SO_4)_3$	5	a)				
$Fe_2(SO_4)_3$	2	10	5	–	2	–
Corrosion product	1.54	10	8	××	8	×××
Corrosion product	0.77	10	6	–	6	–

a) Since the sample containing iron was easily detectable it was pointless to carry out sensory testing.

$$- : P < 95\ \%\ ;\ \times : P \geq 95\ \%\ ;\ \times\times : P \geq 99\ \%\ ;\ \times\times\times : P \geq 99.9\ \%$$

The iron ions present in the distilled water solution in the form of corrosion products were detected by panel members at about the same concentration as those present in the form of added ferric salts. The iron ions dissolved as corrosion products of the carbon steel plates precipitated above a certain concentration. Therefore, the solution used in the sensory tests was first filtered from the precipitate and, if necessary, diluted with pure distilled water. The results of these tests are also shown in Table 1.

In tap water both ferrous and ferric salts formed a red precipitate soon after dissolution, thus these were easily recognizable. The tap water containing as little as 1 mg dm^{-3} ferrous or ferric salt could be distinguished by the naked eye. Thus, these solutions were not subjected to sensory tests.

2.2. Changes in the sensory properties of fruit juices

2.2.1. The effect of ferrous and ferric ions. When tasting fruit juices containing ferrous salts some of the panel members could not differenciate between the three samples even at a concentration of 500 mg dm^{-3}.

Table 2

The effect of various iron salts and of iron contamination present as corrosion product deriving from the carbon steel construction material on the sensory properties of apple juice

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	1 500	10	9	×××	8	×××
$FeCl_2 \cdot 4H_2O$	1 000	10	5	–	5	×××
$FeCl_2 \cdot 4H_2O$ repeated test	1 000	10	10	×××	10	×××
$FeCl_2 \cdot 4H_2O$ repeated test	750	10	5	–	5	×××
$FeSO_4 \cdot 7H_2O$	1 500	10	8	××	8	×××
$FeSO_4 \cdot 7H_2O$	1 000	10	6	–	6	×××
$FeSO_4 \cdot 7H_2O$ repeated test	1 500	10	7	×	7	×××
$FeSO_4 \cdot 7H_2O$ repeated test	1 000	10	6	–	6	×××
$FeCl_3 \cdot 6H_2O$	50	10	8	××	7	××
$FeCl_3 \cdot 6H_2O$	25	10	6	–	6	–
$FeCl_3 \cdot 6H_2O$ repeated test	50	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$ repeated test	25	10	2	–	2	–
$Fe_2(SO_4)_3$	100	10	8	××	8	×××
$Fe_2(SO_4)_3$	50	10	7	×	4	–
$Fe_2(SO_4)_3$ repeated test	100	10	7	×	7	×××
$Fe_2(SO_4)_3$ repeated test	50	10	3	–	3	–
Corrosion product	250	10	10	×××	10	×××
Corrosion product	200	10	5	–	5	×××

For legend see Table 1.

It was surprising that panel members had an astringent, metallic sensation in their mouth for a long time after the test. This is probably due to a delayed reaction.

At higher concentrations where differences were more perceptible, a bitter taste manifested itself in addition to the metallic taste and at 1 500 mg dm^{-3} ferrous ion concentration even a salty flavour was noted. At the concentration of 2 000 mg dm^{-3} ferrous ion panel members could not distinguish between the three samples because of the metallic taste long detained in their mouth. Biscuits or salted sticks did not relieve this sensation.

Results obtained in relation to the apple juice are summed up in Table 2, while those related to sour-cherry in Table 3.

Since experience showed that beverages containing iron ions caused an unpleasant sensation in the mouth if tasted first of the three samples of the triangular test, another evaluation method was given a trial. PIKIELNA (1975) suggests a so-called "fatigue" evaluation method according to which panel members taste the first sample, record their opinion and taste the next sample only after some 15–20 min. The sensation caused by the first sample fades

Table 3

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction material on the sensory properties of sour-cherry juice

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	2 000	10	9	×××	9	×××
$FeCl_2 \cdot 4H_2O$	1 500	10	5	–	5	×××
$FeCl_2 \cdot 4H_2O$ repeated test	12.5	10	7	×	7	×××
$FeCl_2 \cdot 4H_2O$ repeated test	6.25	10	4	–	4	
$FeSO_4 \cdot 7H_2O$	–	–	–	–	–	–
$FeSO_4 \cdot 7H_2O$	1 000	10	2	–	2	–
$FeSO_4 \cdot 7H_2O$ repeated test	25	10	7	×	7	×××
$FeSO_4 \cdot 7H_2O$ repeated test	12.5	10	3	–	3	–
$FeCl_3 \cdot 6H_2O$	150	10	9	×××	7	××
$FeCl_3 \cdot 6H_2O$	100	10	2	–	2	–
$FeCl_3 \cdot 6H_2O$ repeated test	150	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$ repeated test	100	10	6	–	6	×××
$Fe_2(SO_4)_3$	250	10	8	××	7	××
$Fe_2(SO_4)_3$	100	10	5	–	5	×××
$Fe_2(SO_4)_3$ repeated test	250	10	10	×××	10	×××
$Fe_2(SO_4)_3$ repeated test	100	10	5	–	5	×××
Corrosion product	500	10	10	×××	9	×××
Corrosion product	200	10	4	–	4	–

For legend see Table 1.

away by then, but as it is jotted down it may be compared to that of the second one. In Table 3, the data given under "repetition" were obtained by the fatigue method.

2.2.2. The effect of corrosion products. Panel members found that the beverages containing a significant amount of iron originating from corrosion could be detected by their odour and they caused a bitter, stringent, harsh metallic sensation.

The influence of corrosion products on the sensory properties of fruit juices is shown also in Tables 2 and 3.

2.3. Changes in the sensory properties of wines

2.3.1. The effect of ferrous and ferric salts. The study of wines was more difficult than expected. The tests were started soon after vintage and the wine samples were fresh and hard. Thus panel members registered not so much the effect of iron salts but the defects of taste caused by the hardness of the wine. Thus, unambiguous judgement was not obtained.

A further difficulty was caused by the sulfur dioxide used for treating wine. Ferrous salts liberated hydrogen sulfide and the highly unpleasant odour of this covered the effect of iron salts. Panel members noted only this. This may be considered an indirect effect of the iron salts.

Whenever an interfering effect was observed, the evaluation was repeated with new wine samples.

Results obtained so far are summed up in Tables 4 and 5. Because of the intricacy of this question, the study of wines requires further experimental and analytical work.

2.3.2. The effect of corrosion products. As with the fruit beverages, iron contamination was detected by the odour of the wines, too. In wines of higher sulfur dioxide content, the unpleasant odour was that of hydrogen sulfide. Data given in Tables 4 and 5 were obtained in wines of low sulfur dioxide content.

2.4. Changes in the sensory properties of liqueurs

2.4.1. The effect of ferrous and ferric salts. The characteristic golden yellow colour of commercial white butter pear liqueur turned pale upon the addition of ferrous salts. Thus, the samples containing a higher concentration of iron salts could be separated on sight. Samples containing $FeCl_2$ or $FeSO_4$ corresponding to 50 mg dm^{-3} ferrous ion could be easily distinguished and were valued lower because of their unusual colour. At a ferrous ion concentration of 25 mg dm^{-3}, the colour was indistinguishable and some of the panel members preferred the iron-containing samples.

The results of the sensory tests are given in Table 6.

Table 4

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction material on the sensory properties of white table wine

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing samples worse	Significance
$FeCl_2 \cdot 4H_2O$	100	9	7	××	6	××
$FeCl_2 \cdot 4H_2O$	50	7	2	–	0	–
$FeSO_4 \cdot 7H_2O$	200	7	5	×	5	×××
$FeSO_4 \cdot 7H_2O$	100	9	3	–	2	–
$FeCl_3 \cdot 6H_2O$	100	7	7	×××	7	×××
$FeCl_3 \cdot 6H_2O$	50	7	4	–	3	–
$Fe_2(SO_4)_3$	100	7	7	×××	7	×××
$Fe_2(SO_4)_3$	50	7	4	–	3	–
Corrosion product	50	10	10	×××	9	×××
Corrosion product	10	10	6	–	5	×

For legend see Table 1.

Table 5

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction materials on the sensory properties of red wine

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	25	10	8	××	7	××
$FeCl_2 \cdot 4H_2O$	10	10	2	–	1	–
$FeSO_4 \cdot 7H_2O$	100	7	6	××	6	×××
$FeSO_2 \cdot 7H_2O$	50	7	3	–	3	–
$FeCl_3 \cdot 6H_2O$	200	7	7	×××	7	×××
$FeCl_3 \cdot 6H_2O$	100	7	5	×	3	–
$Fe_2(SO_4)_3$	200	7	7	×××	7	×××
$Fe_2(SO_4)_3$	100	7	4	–	3	–
Corrosion product	25	10	8	××	8	×××
Corrosion product	10	10	3	–	3	–

For legend see Table 1.

Table 6.

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction materials on the sensory properties of white butter pear liqueur

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing samples worse	Significance
$FeCl_2 \cdot 4H_2O$	50	10	7	×	7	×××
$FeCl_2 \cdot 4H_2O$	25	10	6	–	6	×××
$FeCl_2 \cdot 4H_2O$ repeated test	50	10	7	×	7	×××
$FeCl_2 \cdot 4H_2O$ repeated test	25	10	6	–	6	×××
$FeSO_4 \cdot 7H_2O$	50	10	10	×××	10	×××
$FeSO_4 \cdot 7H_2O$	25	10	6	–	5	×
$FeSO_4 \cdot 7H_2O$ repeated test	50	10	9	×××	9	×××
$FeSO_4 \cdot 7H_2O$ repeated test	25	10	3	–	3	–
$FeCl_3 \cdot 6H_2O$	50	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$	25	10	5	–	2	–
$FeCl_3 \cdot 6H_2O$ repeated test	50	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$ repeated test	25	10	6	–	6	×××
$Fe_2(SO_4)_3$	250	10	9	×××	8	×××
$Fe_2(SO_4)_3$	100	10	5	–	5	×××
$Fe_2(SO_4)_3$ repeated test	250	10	10	×××	10	×××
$Fe_2(SO_4)_3$ repeated test	100	10	4	–	4	–
Corrosion product	23.2	10	10	×××	10	×××
Corrosion product	11.6	10	6	–	6	×××

For legend see Table 1.

2.4.2. The effect of corrosion products. The colour of the liqueur faded rapidly as an effect of the carbon steel test plates and resembled that brought about by ferrous salts. Preparing the samples for the sensory tests, the liqueur stored with steel plates was diluted with the control liqueur. Results are shown in Table 6.

2.5. Changes in the sensory properties of beer

2.5.1. The effect of ferrous and ferric salts. Both ferrous and ferric salts caused a substantial change in beer. Since commercial beer was used in the experiments, upon the opening of the bottles and adding the iron salts, the major part of carbon dioxide disappeared. Therefore, in order to get comparable results, carbon dioxide had to be removed from the control sample, too. Iron salts caused not only a loss in carbon dioxide, but in most cases also turbidity. In consequence the samples containing iron could be distinguished by the naked eye. The results of the sensory tests are summed up in Table 7.

2.5.2. Effect of iron as corrosion product. When 5 test plates of carbon steel (50 × 100 × 2 mm) were placed in 750 cm³ beer, so much iron was dissolved in 24 h that the beer became unfit for consumption. Hereafter, the test plates were placed for 1 h in the predetermined amount of beer, but even then the iron content was too high. The sensory difference between the control and the samples in which the test plates were immersed for only five minutes was still very highly significant. This sample was then diluted with control beer and the sensory evaluation was carried out in the sample thus prepared. Results are given in Table 7.

Table 7

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction materials on the sensory properties of beer

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	100	10	10	×××	10	×××
$FeCl_2 \cdot 4H_2O$	10	10	5	–	5	×××
$FeCl_2 \cdot 4H_2O$ } repeated test	5	10	7	×	7	×××
$FeCl_2 \cdot 4H_2O$ } repeated test	2.5	10	3	–	3	–
$FeSO_4 \cdot 7H_2O$	10	10	8	××	8	×××
$FeSO_4 \cdot 7H_2O$	5	10	4	–	4	–
$FeSO_4 \cdot 7H_2O$ } repeated test	5	10	8	××	8	×××
$FeSO_4 \cdot 7H_2O$ } repeated test	2.5	10	3	–	1	–
$FeCl_3 \cdot 6H_2O$	5	10	10	×××	10	×××
$FeCl_3 \cdot 6H_2O$	2.5	10	3	–	3	–
$FeCl_3 \cdot 6H_2O$ } repeated test	5	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$ } repeated test	2.5	10	4	–	4	–
$Fe_2(SO_4)_3$	5	10	10	×××	10	×××
$Fe_2(SO_4)_3$	2.5	10	3	–	3	×××
$Fe_2(SO_4)_3$ } repeated test	10	10	9	×××	9	×××
$Fe_2(SO_4)_3$ } repeated test	5	10	6	–	6	×××
Corrosion product	3.9	10	7	×	7	×××
Corrosion product	3	7	7	×××	6	××
Corrosion product	1.73	10	3	–	2	–

For legend see Table 1.

2.6. Changes in the sensory properties of milk

2.6.1. The effect of ferrous and ferric salts. In contrast to the other liquid foods tested, milk got not only a metallic taste from the iron salts but became

Table 8

The effect of various iron salts and iron contamination present as corrosion product deriving from the carbon steel construction materials on the sensory properties of milk

Iron salt	Ferrous or ferric ion concentration (mg dm^{-3})	Number of panel members	Number of those who correctly identified the samples	Significance	Number of those who found the iron-containing sample worse	Significance
$FeCl_2 \cdot 4H_2O$	10	7	7	×××	6	××
$FeCl_2 \cdot 4H_2O$	5	7	5	×	3	–
$FeCl_2 \cdot 4H_2O$ repeated test	10	10	8	××	8	×××
$FeCl_2 \cdot 4H_2O$ repeated test	5	10	5	–	4	×
$FeSO_4 \cdot 7H_2O$	10	7	6	××	6	×××
$FeSO_4 \cdot 7H_2O$	5	7	2	–	2	–
$FeSO_4 \cdot 7H_2O$ repeated test	5	10	9	×××	9	×××
$FeSO_4 \cdot 7H_2O$ repeated test	2.5	10	6	–	6	×××
$FeCl_3 \cdot 6H_2O$	25	10	7	×	7	×××
$FeCl_3 \cdot 6H_2O$	10	7	3		1	–
$FeCl_3 \cdot 6H_2O$ repeated test	10	10	8	××	7	××
$FeCl_3 \cdot 6H_2O$ repeated test	5	10	3	–	3	–
$Fe_2(SO_4)_3$	10	7	6	××	6	×××
$Fe_2(SO_4)_3$	5	7	1	–	1	–
$Fe_2(SO_4)_3$ repeated test	25	10	9	×××	9	×××
$Fe_2(SO_4)_3$ repeated test	10	10	1	–	1	–
Corrosion product	1.52	10	9	×××	9	×××
Corrosion product	1.34	10	9	×××	9	×××
Corrosion product	0.76	10	7	×	7	×
Corrosion product	0.67	10	4	–	4	–

For legend see Table 1.

nauseous. Both ferrous and ferric salts had the same effect. Some of the panel members found the taste of the milk paper-like or foul. Results of these experiments are given in Table 8.

2.6.2. The effect of corrosion products. Results obtained in the vicinity of the threshold values are given also in Table 8.

3. Conclusions

3.1. Changes in the sensory properties of distilled and tap water

The threshold value for the detection of ferrous salts in distilled water is relatively high (100–200 mg dm^{-3}). Since ferrous salts dissolve in distilled water without affecting the colour, they could not be distinguished by the eye.

In contrast, ferric salts affected a change of colour even at a low concentration and thereby promoted differentiation.

Iron ions from corrosion products precipitate from distilled water, probably as an effect of the oxygen absorbed during a longer standing period. Thus, on removing the precipitate from distilled water, the iron content is limited to a low level. Iron ions present in the form of corrosion product were detected at about the same threshold values as ferric salts.

In tap water the major part of iron ions, whether originating from the dissolution of iron salts or present as corrosion products, precipitated, thus sensory evaluation was pointless.

3.2. Changes in fruit juices

A fruit juice of dark red colour and one of a light yellowish brown colour were chosen for the experiments in order to be able to control colour changes in the case of two entirely different fruit drinks.

As an effect of iron salts as well as corrosion products, the smell of the fruit juices became undistinctive. Some of the panel members even noted a metallic smell.

The taste of the fruit juices reacted differently to the different iron salts, however, individual salts affected the two drinks similarly.

Ferrous salts were not detected by triangular testing to a very high concentration level, 1 000–1 500 mg dm^{-3}, in either of the two fruit juices. It was surprising, however, that by the fatigue test ferrous salts were detected at a significantly lower concentration. These results correspond to those found by SHRADER and JOHNSON (1934). This proves that the method of sensory evaluation highly affects the threshold value of detection.

Ferric salts were detectable by the triangular test at a lower concentration than ferrous salts tested by the same method. The differences between the threshold values in the two drinks of the same iron salt and between the threshold values of the two iron salts in the same juice were greater.

Corrosion products affected fruit beverages similarly to ferric salts and the threshold value of their detection was nearer to that of ferric salts.

3.3. Changes in wines

The results obtained in wine tests may be considered only as preliminary information. In the course of these tests, a great number of problems arose which would require elucidation, therefore we wish to give only a few aspects to further work.

It would seem expedient to analyze different types of commercial wines in hard and seasoned condition as a function of their sulfur dioxide content, and the effect of iron content upon the sensory quality during the aging of the wine.

3.4. Changes in liqueurs

Of the liquid foods studied, the only one the colour of which was more sensitive to ferrous salts than ferric salts, was liqueur. The colour of the liqueur containing 25 mg dm^{-3} ferrous ion resembled that of the control. Inspite of this, most panel members could easily tell the two identical samples from the third, they preferred, however, the contaminated samples. Since, in contrast to other liquid foods studied, this liqueur contains 40% of sugar, the taste-modifying effect of iron salts was advantageous because of the high masking capacity of the sugar content.

Another characteristic differing from that of other liquid foods is that the threshold value for $Fe_2(SO_4)_3$ is four times higher than for other salts.

The threshold value for corrosion products lies in the same region as with ferrous salts and the liqueur reacts to corrosion products just as to ferrous salts. As regards its sensory properties, commercial pear liqueur is rather sensitive to iron contamination. A further proof of this observation is that, if not immediately upon dissolution, but certainly during storage, ferrous ions bleach the liqueur even at a low concentration level (10–25 mg dm^{-3}). Therefore, great care has to be taken during manufacture to avoid contamination by iron.

3.5. Changes in beer

Investigations have shown beer to be extremely sensitive to iron contamination. Although threshold values for the detection of ferrous and ferric salts were modified in the course of repeated tests it was possible to establish the boundaries within which iron significantly reduces the sensory quality of beer. It is of interest to note that, of all the iron contaminations tested, corrosion products may be detected at the lowest concentration in beer. Thus the requirement suggested by CASH (1975) to limit the iron concentration in beer at 0.25 ppm seems indicated.

3.6. Changes in milk

Milk is rather sensitive to iron contamination, too. The threshold value for iron salts was about the same as with beer, and iron coming from corrosion products was detectable at concentrations *ca.* ten times lower.

The threshold values found correspond to those reported by DAVIES (1936), but the threshold value for iron present as corrosion product was substantially lower. The oxidative effect of iron contamination further degrades sensory properties.

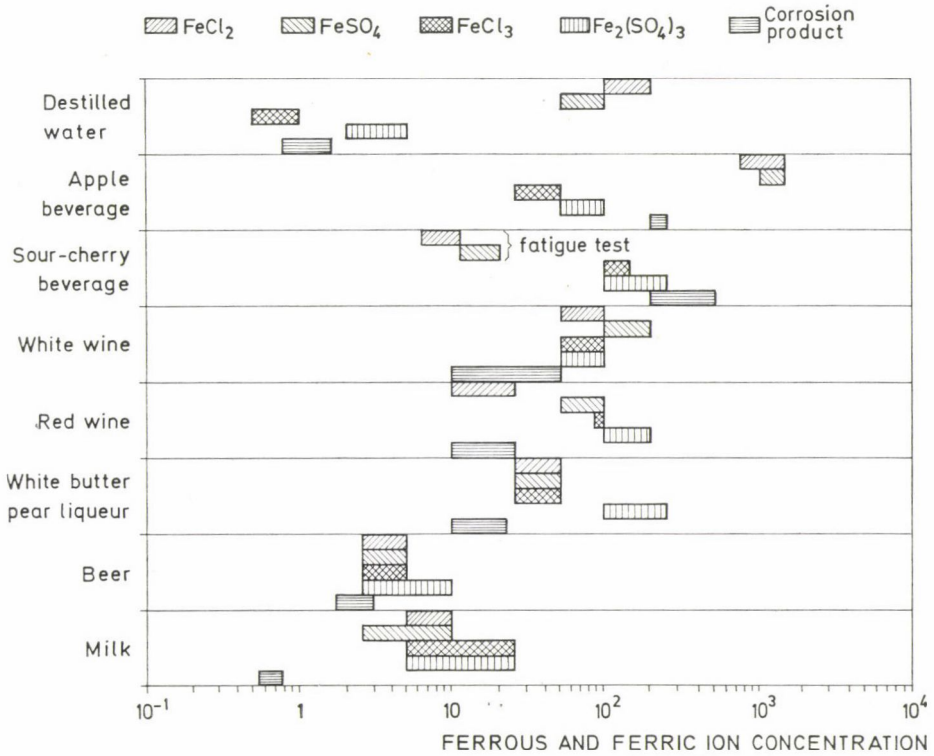

Fig. 1. Threshold values of detecting ferrous and ferric ions originating from added iron salts or corrosion products, in liquid foods

In summary, it may be concluded that the liquid foods investigated fall into three groups: on the basis of their sensory properties fruit juices are the least sensitive, wines and white butter pear liqueur are of medium sensitivity, while beer and milk are very sensitive. This is illustrated in Fig. 1.

Except for fruit beverages, all the foods investigated were most sensitive to iron contamination coming from corrosion products and this became apparent in the change of their sensory properties.

The effect of ferrous and ferric ions on the sensory properties differed substantially independently from the anions present.

With identical iron ions, the effect of anions was less important.

The analysis of sour-cherry juice has shown that the method used for sensory evaluation highly affected the threshold value of detection. This phenomenon is probably due to the fact that iron ions, particularly if present at low concentration, cause a delayed sensation in the mouth.

In the present study, tests were carried out only with freshly prepared iron solutions, thus the changes caused by iron ions during storage were not investigated.

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SHELF LIFE EXTENSION OF MUSHROOMS (*AGARICUS BISPORUS*) BY GAMMA IRRADIATION

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Fresh mushrooms were irradiated (0, 1.0, 2.5 kGy) and kept for 8 days at 287 K (14 °C). The stored samples were studied for different quality parameters. It was observed that the growth rate was higher in the pileus than in the stipe portion of mushrooms. Veils started unstretching on 1st day, and on 2nd day all were open in the control samples. An irradiation dose of 2.5 kGy strongly inhibited the veil opening throughout the storage period. Highly significant moisture loss occurred during storage. Irradiation significantly reduced the weight loss after 2 days of storage. Highly significant softening occurred in storage, but irradiation improved this condition. The irradiation dose of 2.5 kGy showed better results than 1.0 kGy.

Cultured mushroom is a highly perishable vetegable, and can be kept in prime condition only for 1 day at 283 K (10 °C). Cell division continues after harvest. During storage, the pileus and stipe expand. Such changes make mushrooms unattractive. Much research has been carried out over the last many years for extending the storage life of mushrooms. Antioxidants, prepacking and low storage temperature have been used to maintain mushrooms in good condition (GORMLEY, 1975). But all of these techniques have been moderately successful. STADEN (1965) was the first investigator who was able to extend the storage life of mushrooms by ionizing radiation. After that time, different workers tried this method for mushroom preservation. The irradiation doses which were used by them varied considerably. The 0.1 kGy was the lowest limit (GILL *et al.*, 1969), followed by 0.25–1.0 kGy (STADEN, 1965; KOVÁCS & VAS, 1974), between 1.0–3.0 kGy (YAMAGUCHI & CAMPBELL, 1973; STANEK, 1978) and 2.0–3.0 kGy (SKOU *et al.*, 1974; SALKOVA *et al.*, 1978).

The review of the literature shows that there are discrepancies concerning the effects of irradiation on the physiological parameters of stored mushrooms. The present work is, therefore, undertaken to find a suitable irradiation dose for longer storage life of mushrooms when these are stored at a relatively high temperature (287 K) (14 $\pm$ 2 °C).

1. Materials and methods

1.1. Material

Agaricus bisporus (L.) was used in the present investigations. The raw material for the examination was obtained from the MUSHROOM PRODUCTION UNIT of the DUNA AGRICULTURAL COOPERATIVE, Budapest. The fresh mushrooms were harvested at the stretched-veil stage having a diameter of 4–5 cm. The samples were sorted for healthy fruit bodies, trimmed from the base, and soil was brushed off. Radiation treatment of mushrooms was carried out in the ^{60}Co panoramic source of 3.1 PBq activity at the experimental plant of the INSTITUTE of ISOTOPES of the HUNGARIAN ACADEMY of SCIENCES. Irradiation doses of 1.0 and 2.5 kGy were applied, while one-third of the mushrooms was used as the non-irradiated control. Irradiated and unirradiated mushrooms were stored at 14 $\pm$ 2 °C, and 80–90% relative humidity and studied after 0, 1, 2, 4, 6 and 8 days for different quality parameters.

1.2. Examination of texture

A *Texturemeter* (ZENKEN Co., Japan) was used to perform textural profile studies. The procedure reported by KOVÁCS and VAS (1969) was adopted for these investigations.

Texturemeter tests were carried out with mushroom pileus and stipe portions separately. A cylinder of about 2.0 cm was cut from each of the 10 mushrooms of each treatment and trimmed to a height of 1.0 cm keeping close to the outer surface. The tooth-shaped brass plunger was used for the experiments. The maximum space between the moving horizontal head and the flat plate of the load cell was 4 cm. The recorder chart was driven at a speed of 750 mm per min. Hardness was measured as the height of the peak.

1.3. Veil, pileus diameter and stipe elongation

Immediately after harvest, mushrooms were cleaned and sorted into three lots of 20 mushrooms each for treatments with 0, 1.0 and 2.5 kGy. Pileus diameter and stipe length (in mm) of the selected samples were determined in the fresh state and then stored up to 8 days. Changes in pileus diameter and stipe length during storage were measured using vernier caliper. The stored mushroom samples were rated visually for the extent of veil opening and were divided into fully closed, partly opened and fully opened groups. Increase in diameter of pilei and length of stipes and the state of veil were calculated as percentages of the original values.

1.4. Determination of weight loss

Freshly harvested unirradiated and irradiated mushroom samples were sorted for uniform size and divided into 12 lots. Each lot contained about 10 mushrooms. The experimental samples were held up to 8 days in wax-coated paper boxes. All lots of the mushrooms were weighed daily, difference in weight was calculated and loss was expressed as percentage of the original values.

2. Results

2.1. Visual observations

Freshly harvested mushroom samples had closed caps and were white and attractive in colour. Storage studies revealed that the non-irradiated samples deteriorated most rapidly. It was observed that on the 1st day of storage the veil started opening and during the 2nd day all the mushrooms were with completely broken veils exposing the dark coloured gills and spores were being shed out in control samples. The white caps and stem became brownish in

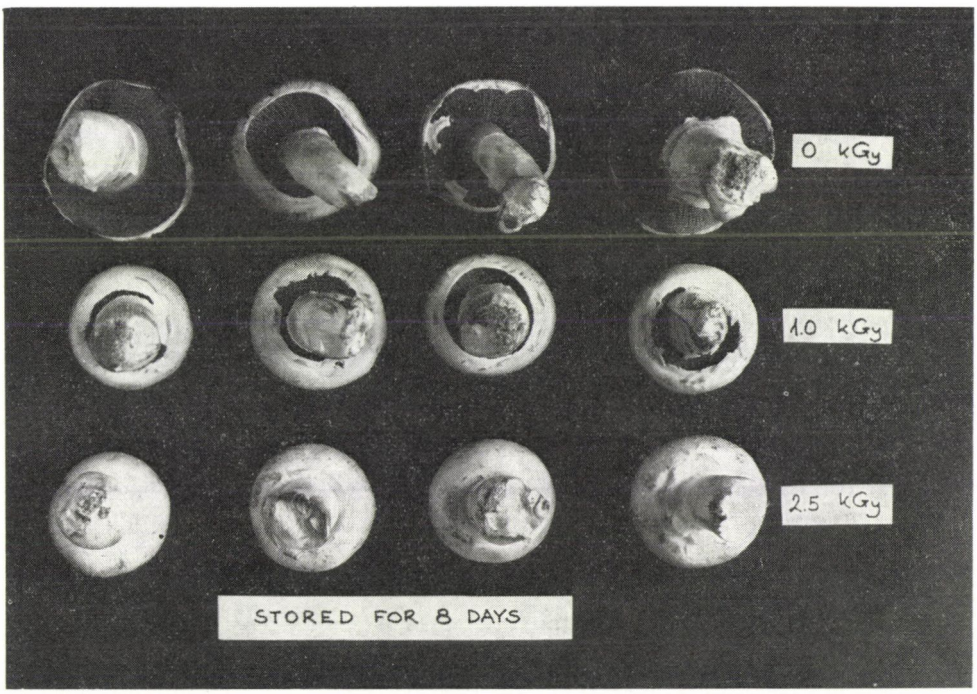

Fig. 1. Effect of ionizing radiation on the keeping quality of *Agaricus bisporus* stored for 8 days
(Temp.: 287 K (14 ± 2 °C), R. H.: 80–90%)

colour. On the other hand, mushrooms which had received dosages of 1.0 and 2.5 kGy were in good condition. The irradiation dose of 2.5 kGy was found to be more effective as compared to 1.0 kGy in inhibiting the growth of mushrooms during the storage period of 8 days (Fig. 1).

2.2. Veil, pileus expansion and stipe elongation

The state of the veil, pileus expansion and stipe elongation have been considered as the critical quality parameters of mushrooms. The percentual changes in the growth rate of mushrooms and in cap opening are shown in Table 1 and Fig. 2.

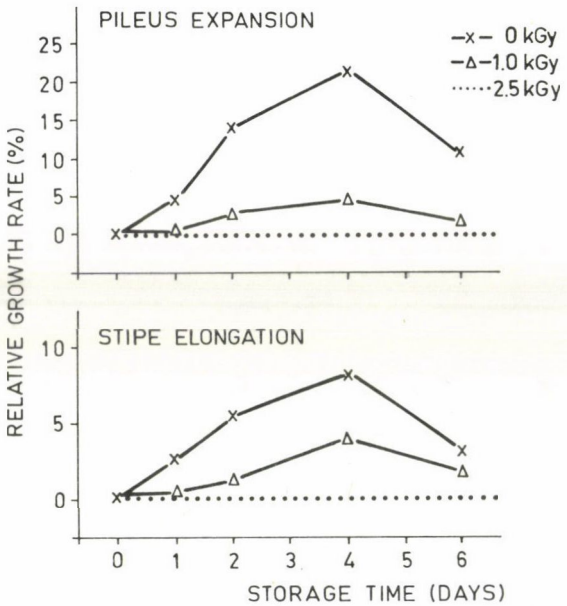

Fig. 2. Effect of irradiation on the relative growth rate of mushrooms as a function of storage time
(Temp.: 287 K (14 ± 2 °C), R. H.: 80–90%)

The results of the experiment showed that the non-irradiated stored mushrooms deteriorated most rapidly. Pilei had completely expanded, stipe had elongated, veil fully opened and spores were being shed out from the gills. The veils which were completely closed on 0 day attained the 100% partly opened state, and 100% fully opened state on the 1st and 2nd days of storage in control samples. On the other hand mushrooms which had received irradiation dosages of 1.0 and 2.5 kGy were in good condition.

The increase in relative growth rate was quite high in unirradiated stored samples. The growth rate in control mushrooms was linear and gradual at

Table 1

Effect of storage and irradiation on the state of the veil in mushrooms at 287 K (14 ± 2 °C); relative humidity 80-90%

Treatment	Storage time (day)	State of the veil		
		Fully closed[a] (%)	Partly opened[a] (%)	Fully opened[a] (%)
0 kGy	0	100	0	0
	1	0	100	0
	2	0	0	100
	4	0	0	100
	6	0	0	100
	8	0	0	100
1.0 kGy	0	100	0	0
	1	100	0	0
	2	25	75	0
	4	10	90	0
	6	0	100	0
	8	0	100	0
2.5 kGy	0	100	0	0
	1	100	0	0
	2	100	0	0
	4	100	0	0
	6	80	20	0
	8	80	20	0

[a] Based on observations on 20 caps (n = 20)

first and then attained a maximum limit on the 4th day. The rate of pileus expansion and stipe elongation seems to increase or decrease in the same way (Fig. 2) and observation of the individual samples showed that both ceased growing at approximately the same time. Growth was completely stopped by the irradiation treatment of 2.5 kGy. At 1.0 kGy treatment samples were found intermediate between control and 2.5 kGy irradiated mushrooms.

Delay in ripening or senescence, which has been measured by the state of veil is regarded as one of the most important considerations ever since STADEN (1965) published his first paper on irradiation preservation of mushrooms. All the numerous investigations which have been carried out up till now are concerned with this effect of delay in senescence. Increase in pileus diameter causes the veil to break and expose the gills which results in loss of freshness. The expansion of pileus and elongation of stipe are markedly suppressed by irradiation. Generally all investigators agreed with this, but workers

reported different dose values for this purpose (GILL *et al.*, 1969; SALKOVA *et al.*, 1978). At 0.25 kGy SKOU and his co-workers (1974) observed a significant but transitory effect which improved at doses up to 2.0 kGy, the state of the veil of mushrooms. STANEK (1978) noticed a low effect with 0.5 kGy, while irradiation doses of 1.0–3.0 kGy were found significant on mushroom veil opening at 288 K (15 °C) during storage period of 10 days. As temperature and relative humidity seriously affect the opening ability of the veils (LANGERAK, 1972), the prevailing varied experimental conditions may explain the reported varying doses of irradiation mentioned in the literature. Generally it has been agreed by all investigators that all radiation doses inhibit veil opening and this effect is improved with higher doses.

Pileus expansion and stipe elongation in mushrooms take place during their storage and the growth rate depends upon the temperature and environmental conditions of the store house (KOVÁCS & ZUKÁL, 1978). Under different storage conditions, investigators noticed different radiation doses suppressing the growth rate of mushrooms. GILL and his co-workers (1969) found significant retardation in pileus expansion and stipe elongation at irradiation doses of 1.0 kGy or above, and YAMAGUCHI and CAMPBELL (1973) noted that a dose of 1.0 kGy was more effective than 0.5 kGy. SKOU and his co-workers (1974) observed a significant effect on pileus and stipe growth above 1.0 kGy.

2.3. Texture

The results obtained from the experiment showed (Fig. 3) that both the pileus and stipe portions of mushroom became soft during storage. At the beginning of the experiment, the pileus portion had higher values, about double that of the stipe, but after 15 days of storage both portions gave somewhat similar values of texture. A statistically significant ($P < 0.05$) softening was observed in both portions of mushroom during the storage period of 15 days.

Contrary to the general opinion about softening induced by irradiation, the findings of the present investigation indicated that irradiated mushrooms were better (harder) in texture than their control samples. In the pileus portion no immediate effect of irradiation (1.0 and 2.5 kGy) was observed on 0 day. On the 4th day of storage the pileus of the unirradiated mushrooms exhibited statistically highly significant ($P < 0.01$) differences in softening as compared to samples irradiated at 1.0 and 2.5 kGy. The latter two showed no significant difference between themselves. Similarly, there was no significant difference in texture between irradiated mushrooms stored for 0 and for 4 days. During further storage, all the three treatments (0, 1.0 and 2.5 kGy) gave statistically highly significant differences ($P < 0.01$), the control presenting soft and 2.5 kGy giving the high, while 1.0 kGy treatment showing the intermediate values of texture. The effect of storage and irradiation on the stipe was similar to that

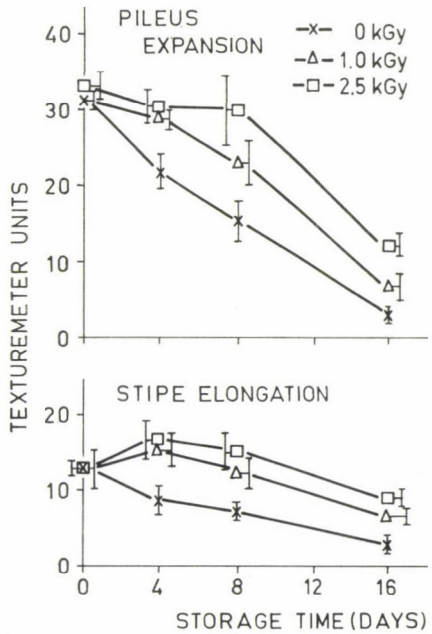

Fig. 3. Effect of irradiation on the texture (hardness) of mushrooms as a function of storage time
(Temp.: 283 K (10 ± 2 °C), R. H.: 80–90%)

on the pileus portion, except that on the 4th day the textural values of irradiated mushrooms were higher than those of the freshly harvested mushrooms.

Only a few authors presented results concerning the effect of irradiation on the texture of mushrooms, probably because of their preoccupation with the growth and state of veil which have been mentioned in the previous section. MURR and MORRIS (1975) reported that temperature and duration of holding influenced the textural characteristic of fresh mushrooms. GILL and his co-workers (1969) showed that texture, when measured by shear press, remained unaffected at doses below 2.0 kGy. KOVÁCS and VAS (1974) did not observe any change in texture either immediately or after irradiation in mushrooms, when samples were tested by organoleptic tests.

It was found in the present experiments that toughness in mushrooms decreased during all the storage period of 16 days. There can be two reasons responsible for this softening: (*a*). The differences in texture during the storage are due to changes in cell wall structure. Cell turgidity has been considered as the main factor determining the textural qualities of food (MATZ, 1962). If this applies to the toughness of mushrooms, then it is likely that the instrumental values for hardness will drop when mushrooms loose water in storage which, of course, may happen frequently. (*b*) Cell autolysis the rate of which increases during senescence, causes a decrease in toughness, and it is well known that

most of the fungi undergo a certain degree of autolysis (VILLANUEVA, 1966). Irradiation treatment inhibits or slows down the life process in mushrooms due to which no or less expansion takes place and so less moisture is lost, and the cell remains in a better state. In this way irradiated mushrooms exhibit a far better texture than the control samples.

2.4. Loss of moisture

It was found that the weight loss of the control increased from 0 to 61% during the storage period of 8 days (Fig. 4). The weight loss was about 61, 47 and 45% on the 8th day of storage in the 0, 1.0 and 2.5 kGy treatments, resp. In all the cases highly significant losses ($P < 0.01$) occurred during storage. No statistically significant difference was observed in weight loss due to irradiation treatments till the 2nd day of storage ($t = 0.961$), but during later storage, highly significant differences in weight were noted in unirradiated mushrooms as compared to irradiated samples. This may be partly due to veil opening, pileus expansion and stipe elongation, which produced more surface area for moisture evaporation. No significant difference in the effects of irradiation treatments of 1.0 and 2.5 kGy in respect of moisture loss was seen in mushrooms stored until the 2nd day, after which both treatments also differed significantly at the 95% probability level.

The finding of GILL and his co-workers (1969) revealed that irradiation resulted in higher moisture losses in mushrooms but they suspected that higher values were due to one mushroom which lost 33 % of its original weight. YAMAGUCHI and CAMPBELL (1973) found that moisture losses increased in storage at 283 K (10°C), but did not detect any significant loss due to irradiaton. They reported that only non-irradiated mushrooms showed significant decrease in dry weight after 6 days of storage. SKOU and his co-workers (1974) recorded

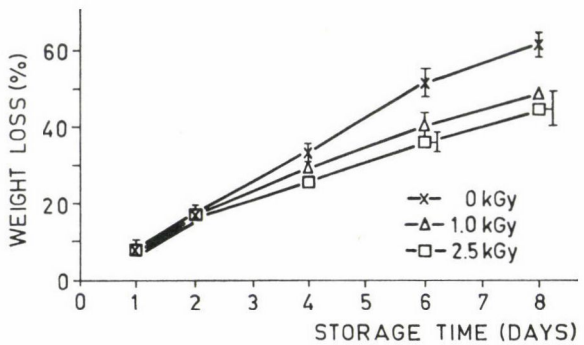

Fig. 4. Effect of irradiation on the weight loss of mushrooms as a function of storage time (Temp.: 287 K (14 ± 2 °C), R. H.: 80–90%)

a weight loss of about 15 and 25 % in irradiated and control mushrooms for 10 days of storage at 283 K (10°C) and 90—95 % relative humidity.

Mushrooms have porous sponge-like structure. During storage they dehydrate under conditions of low humidity. Moisture conditions are, therefore, believed to influence the storage quality of mushrooms, which are highly sensitive to practically any level of relative humidity. Temperature, packaging and an air flow during storage play additional roles in this respect (LANGERAK, 1972). These considerations may be the reasons for the discrepancies reported in the literature.

3. Conclusion

The present investigation suggested that irradiation treatment of 2.5 kGy is necessary for a satisfactory shelf life extension of mushrooms at 287 K (14 ± 2 °C) and 80–90% relative humidity. Veil opening is very sensitive to irradiation. The inhibition of veil opening and retardation of growth in pileus and stipe depend on the irradiation dose. Moisture and textural qualities of mushrooms are also improved by irradiation. Weight loss and texture are indirectly influenced since these are directly related to the retarding effect of irradiation on growth and ripening. But as irradiation cannot completely stop the loss of moisture from the mushrooms, practically it becomes necessary, from the marketing point of view, to use some type of packaging material *e.g.* perforated foil, *etc.* to reduce the moisture losses.

The quality of mushrooms cannot be defined by one or two factors. Maturity stage, distribution time, type and tightness of packaging, container style and various other factors influence the keeping quality of mushrooms. The apparently favourable effect of irradiation increases with increasing temperature and changes in relative humidity. So a definite universal dosage cannot be proposed but in each marketing trial an appropriate dose should be selected in order to take advantage of minimum irradiation treatment with maximum benefit under different circumstances.

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DISINTEGRATION OF VEGETABLE TISSUES AS A FUNCTION OF POLYGALACTURONASE CONCENTRATION AND INCUBATION PERIOD

K. ZETELAKI-HORVÁTH

(Received 12 December 1979; accepted 27 January 1980)

Changes in particle size distribution of six vegetables disintegrated by endo-polygalacturonase were investigated (4 enzyme concentrations, 3 incubation periods, temperature: 50 °C).

For each vegetable, the size range in which the frequency of the tissue particles was highest was found to be generally identical at every enzyme concentration. In most cases, the peaks of frequency distribution were observed at identical particle sizes with different maximum values. In certain cases, however, increasing the enzyme concentration shifted the peak toward smaller particle sizes (*e.g.* winter squash).

With carrots, kohlrabi and winter squash, particle size decreased with increasing enzyme concentration (from 0.06 to 0.6%).

On comparing the position and maximum values of the peaks, disintegration to smaller sizes could be intensified by increasing the enzyme concentration.

In the case of green paprika, potato and celery, however, increasing the enzyme concentration did not result in a substantial increase in the frequency of smaller particles.

With some of the vegetables (*e.g.* celery and kohlrabi), the extension of the incubation period from 1 to 3 h shifted the frequency of particle size distribution towards the higher ranges (30–40, 50–60 μm), permitting the conclusion that the extension of the incubation period apparently led to some aggregation.

The frequency maxima for the individual vegetables were as follows:

carrots: 10, 60 μm; celery: 40, 60 μm; green paprika: 10, 60 μm; kohlrabi: 10, 40 μm; squash: 30 and 70 μm.

The standard deviation of results for a given vegetable was below 1% when processed immediately, about 2.5% for repeated treatments.

The average standard deviation for the granulometric data of all the different vegetables at all treatments was about 10–15%.

In research work carried out at the CENTRAL FOOD RESEARCH INSTITUTE, Budapest over several years, we succeeded in developing a fermentation technology to produce endo-galacturonase (ZETELAKI-HORVÁTH & BÉKÁSSY-MOLNÁR, 1975; ZETELAKI-HORVÁTH, 1978).

The utilization of this enzyme in the food industry was also studied. In the course of the experiments, it was established that endo-polygalacturonase is capable of disintegrating vegetable tissues into individual cells or cell agglomerates below 200 μm (ZETELAKI-HORVÁTH & GÁTAI, 1977a). The extent of disintegration is highly affected by the parameters of treatment, the activity of the enzyme applied, the quantity of the enzyme, the treatment period, the

biochemical composition of the treated tissue, the anatomy of the plant and its varieties.

In the course of the preliminary experiments, the characteristic particle sizes of some vegetables were also determined by the sedimentation technique (ZETELAKI-HORVÁTH & URBÁNYI, 1978).

It is assumed that the stability of vegetable juices and vegetable-fruit cocktails depends on the particle size of the tissue suspensions obtained by enzymatic disintegration. Vegetable juices containing smaller particles take a longer time to settle, are more stable than those containing larger particles.

The present study is an investigation into the particle size of various vegetable juices as influenced by the endo-polygalacturonase (endo-PG) concentration and the period of treatment. In the case of carrot, different varieties were tested and compared.

1. Materials and methods

1.1. Enzyme preparation

An enzyme preparation obtained from *Asp. awamori* and sterilized by gamma radiation at the Central Food Research Institute, Budapest (ZETELAKI-HORVÁTH & KISS, 1978) was used in the experiments. The activity of the preparation was 3 000 l h^{-1} g^{-1} determined by viscosimetry (ZETELAKI-HORVÁTH & VAS, 1972).

1.2. Vegetable varieties and treatments

Six vegetables: carrots [varieties: *Chantenay*, *Fertődi vörös* (Red of Fertőd) and *Vörös óriás* (Red giant)]; kholrabi: *Szentesi kék* (Blue of Szentes); celery: *Imperator*; potato: *Rózsa* (Rose); green paprika: *Keszthelyi fehér* (White of Keszthely) and winter squash: *Nagydobosi*, were studied at endo-PG concentrations: 0.06, 0.12, 0.6, 1% and an incubation period of 3 h or at incubation periods of 1, 2 and 3 h and 0.5% endo-PG concentration, resp. Following disintegration, the particle size distribution was determined by the sedimentation technique.

The juice fractions used in the measurements were prepared by passing them through sieves of 1 mm and 250 μm (ZETELAKI-HORVÁTH & GÁTAI, 1977a).

(The majority of the vegetable varieties was kindly placed at our disposal by the Tordas section of the NATIONAL INSTITUTE for AGRICULTURAL VARIETY TESTING.)

1.3. Sedimentation technique

Endo-PG disintegration was carried out in three parallels and the average of these was used as a sample. The cells were obtained by centrifuging and washing in distilled water 3 times. Then a suspension of about 0.15% solids content was prepared and 90 cm^3 were applied to 3 sedimentation columns. Ten-cm^3 samples were taken at intervals corresponding to 230, 150, 110, 80, 60, 42, 28 and 16 μm particle size diameter.

The weights measured permitted calculating granulometric data and, from the granulometric curves based on the latter, the cumulative frequency and the frequency distribution were calculated by interpolation.

The granulometric data are the average values of the fractions of three parallel sedimentation columns. The data obtained with enzyme concentrations of 0.06, 0.12 and 0.6% represent the averages of two parallel measurements for the following 4 vegetables:

carrots: *(Chantenay, Vörös óriás);*
potato: *(Rózsa);*
kohlrabi: *(Szentesi kék);*
squash: *(Nagydobosi).*

In the study of the effect of incubation period on the particle size, another carrot variety, *Fertődi vörös*, was used.

2. Results

2.1. Study of carrots

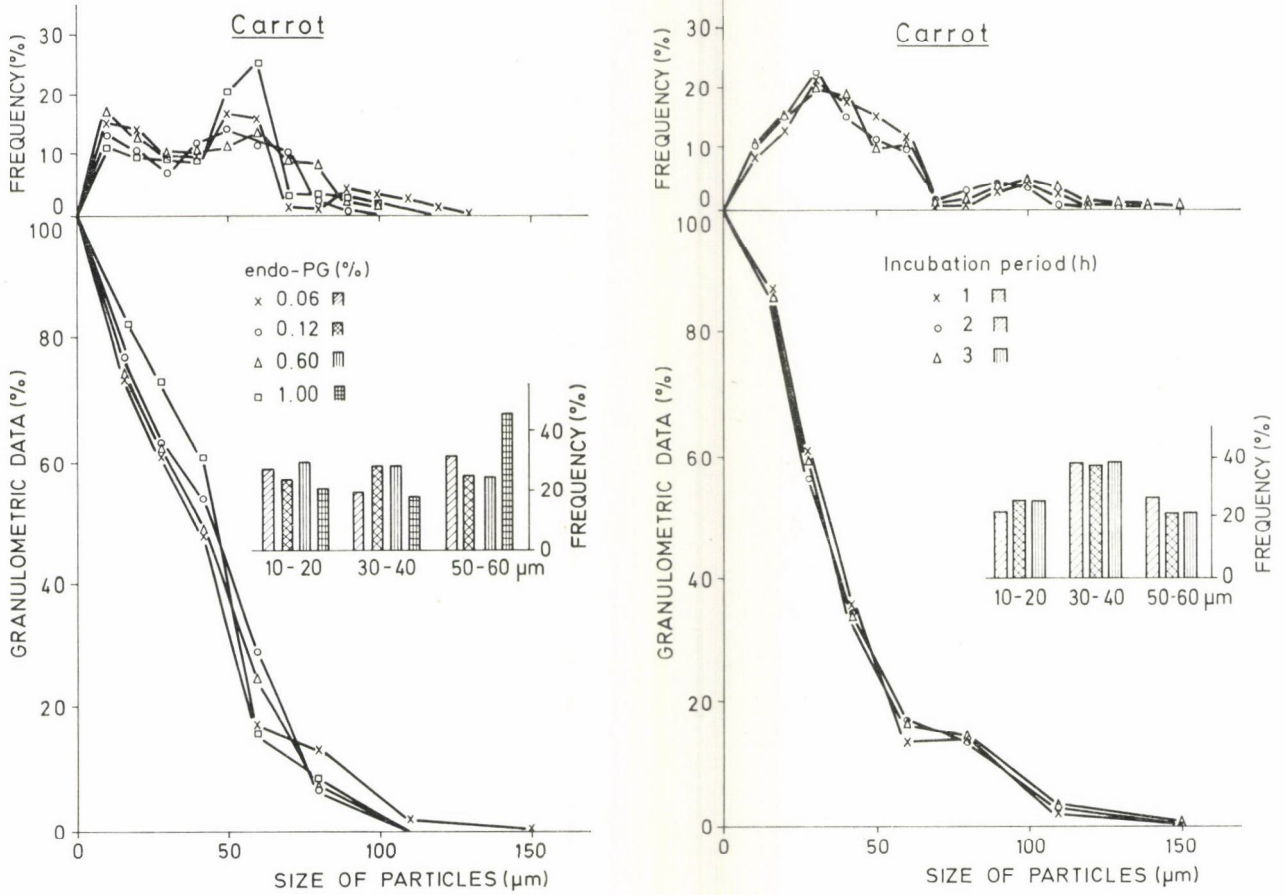
In the course of the study on carrots, frequency peaks were observed in two size ranges: at 10–20 and 50–60 μm. Particles of 10 μm were most numerous in the sample containing 0.6% endo-PG, while particles of 50–60 μm were most frequent with the highest enzyme concentration (Fig. 1).

On the basis of the frequency distributions of particle sizes, the addition of enzyme at a concentration higher than 0.6% seems to be superfluous.

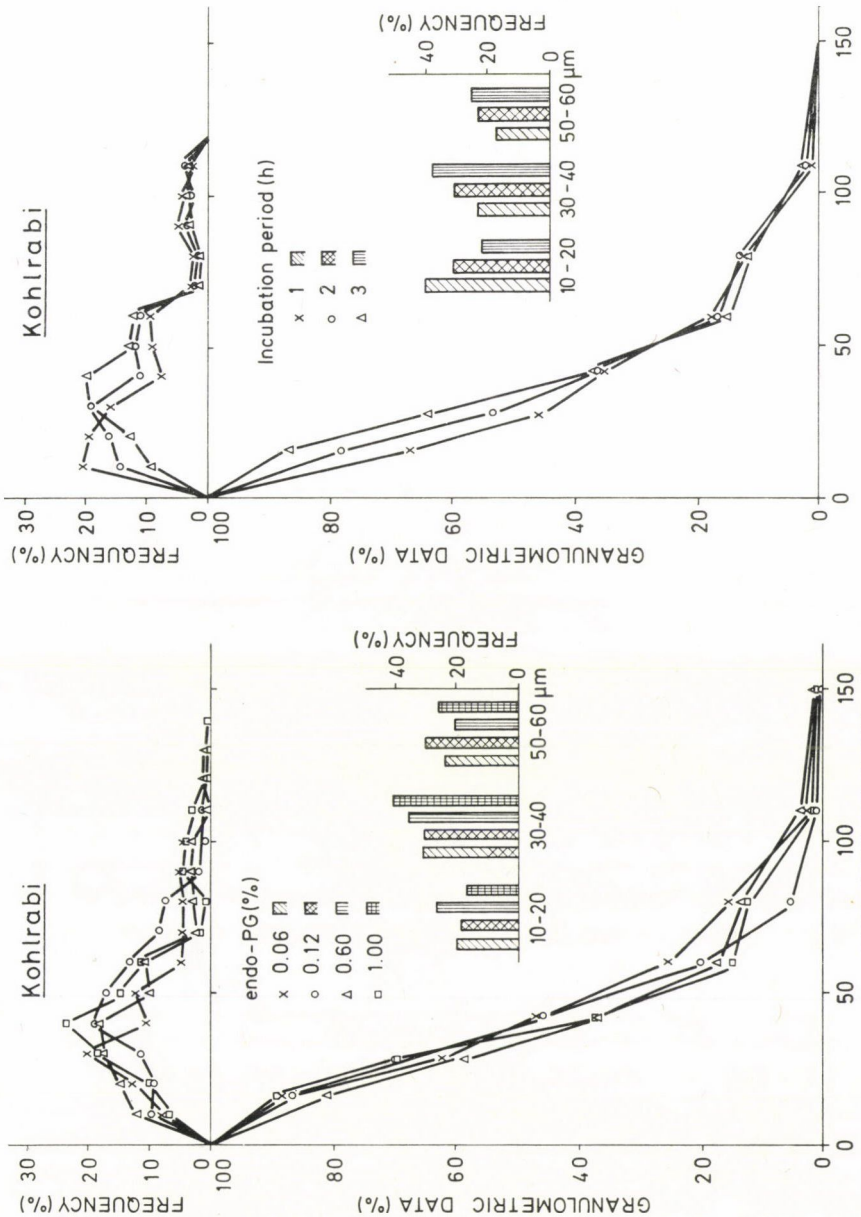
In disintegration with 0.5 %endo-PG, variation of the incubation period (1, 2 or 3 h) did not seem to bring about differences in the 10–40 μm range (Fig. 2).

The frequency of particles in the size range of 10–40 μm did not change upon extending the incubation period from 2 to 3 h.

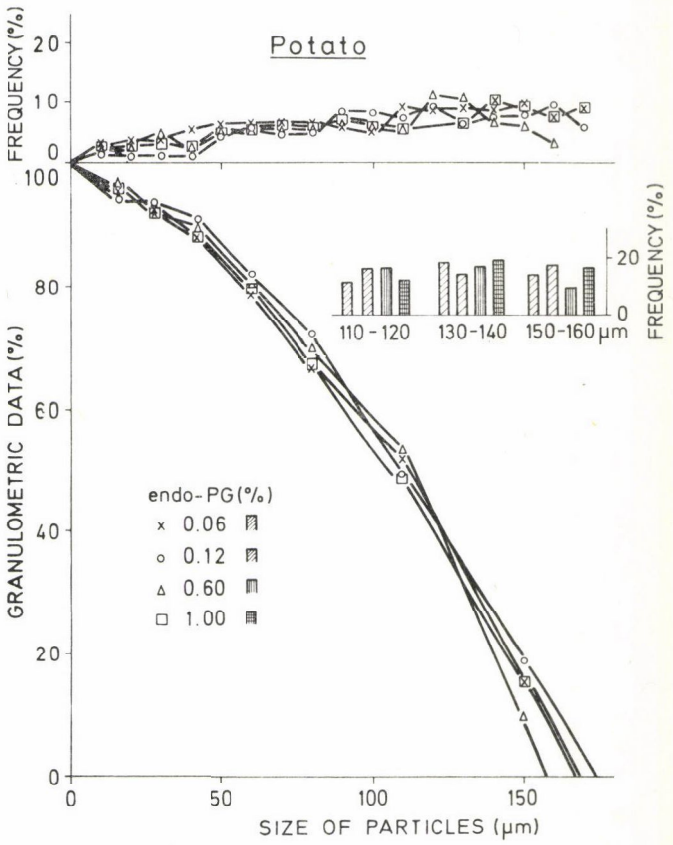
The quantity of particles of 10–20 μm after 1 h incubation period was only 4% less than after 2 h.

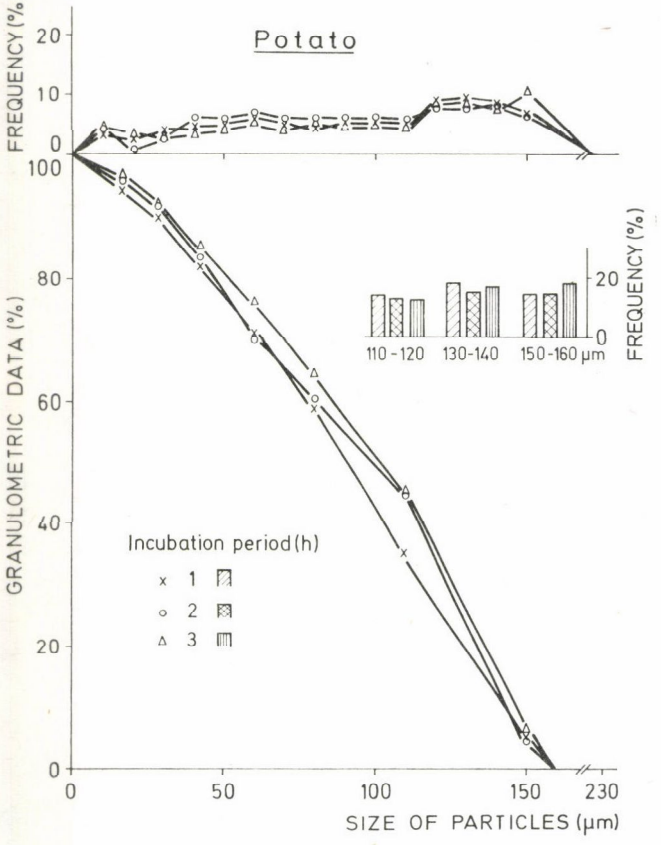


Figs. 1. and 2. Granulometric data and frequency distribution of particle size for carrots (two varieties: *Chantenay*, *Fertődi vörös*), treated for 3 h at different endo-PG concentration levels or for different incubation periods with 0.5% endo-PG (variety: *Vörös óriás*)



Figs. 3. and 4. Granulometric data and frequency distribution of particle size for kohlrabi treated for 3 h with different endo-PG concentrations or for different incubation periods at 0.5% enzyme concentration (two repetitions with the same variety: *Szentesi kék*)





Figs. 5. and 6. Granulometric data and frequency distribution of particle size for potato treated with different endo-PG concentrations for 3 h or with 0.5% enzyme for different incubation periods (two repetitions with the same variety: *Rózsa*)

2.2. Study of kohlrabi

With kohlrabi *(Szentesi kék)* frequency peak was at 30 μm in the sample treated with 0.6% enzyme and at 40 μm in those treated with 0.12, 0.6 and 1.0% enzyme, resp. (Fig. 3).

Particles of 10–20 μm were most frequent in samples treated with 0.6% enzyme, but particles of 30–40 μm were also present in nearly the highest quantity.

In the study of the effect of incubation period, particles of 10–20 μm were found to be most frequent after a 1 h period. With increasing incubation period the quantity of particles of 10–20 μm gradually diminished and those of 30–40 and 50–60 μm increased. This phenomenon appears to indicate aggregation. Taking this into consideration, an incubation period longer than 1 h is not recommended with kohlrabi (Fig. 4).

2.3. Study of potatoes

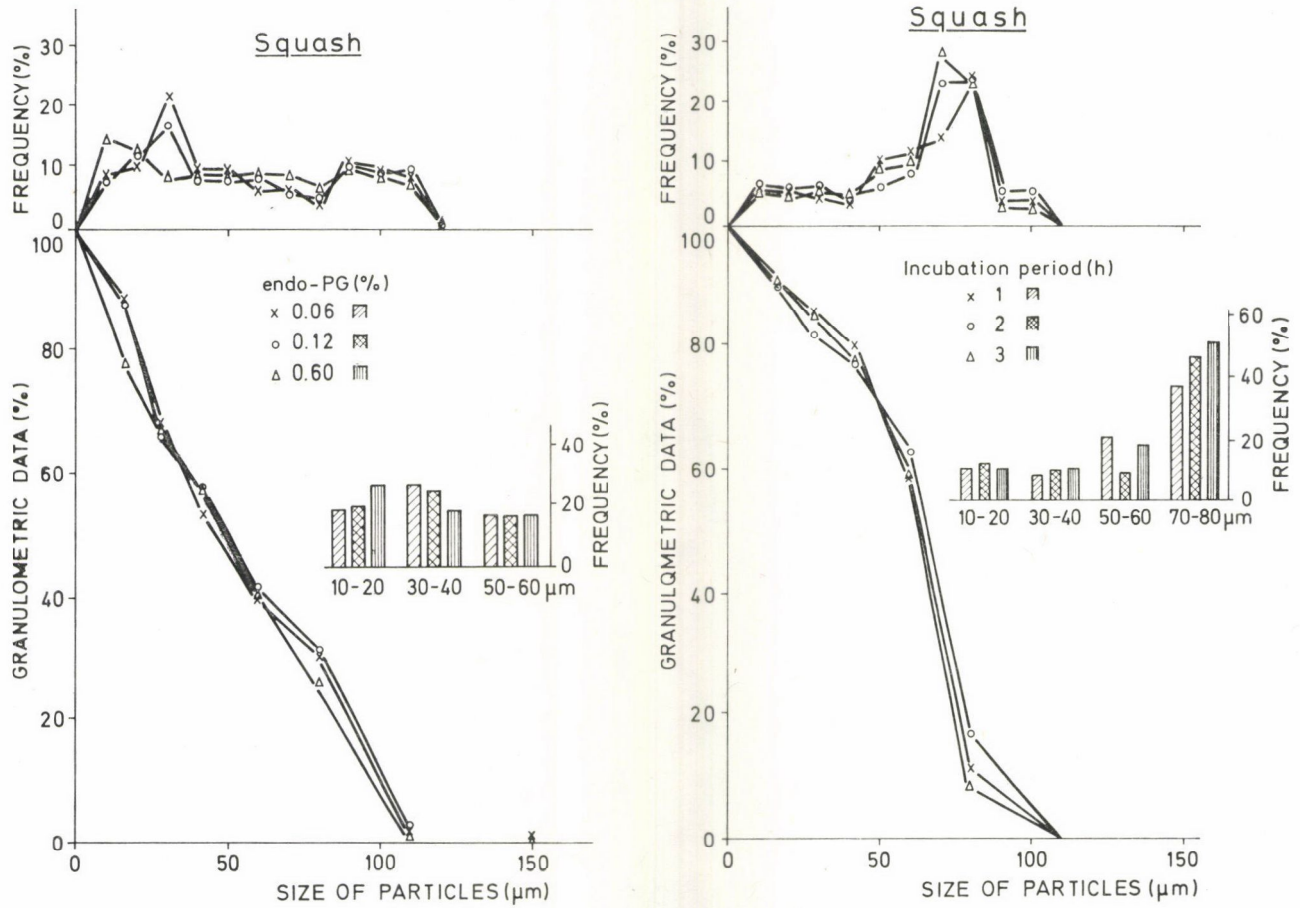
Potato variety *Rózsa* was treated at four different enzyme concentrations and the frequency peaks of particle size were found in the size range of 110–140 μm. The highest quantity of particles of 110–120 μm were in the sample treated with 0.6% endo-PG and the nearest amount was in the sample containing 0.12% enzyme. Taking into account, however, particle sizes of 130–140 μm, too, the application of 0.6% endo-PG seems more advantageous (Fig. 5).

Also with potatoes, treatment for a period longer than 1 h is not indicated. The amount of particles belonging to both ranges, 110–120 and 130–140 μm, was highest in samples treated for 1 h only (Fig. 6).

2.4. Study of squash

The squash variety *Nagydobosi* was treated with 3 endo-PG concentrations (0.06, 0.12 and 0.6%). The most frequent particle size was 30 μm with the lowest and medium concentrations, and 10 μm for the highest enzyme concentration. This means that, with the lower enzyme concentrations the squash tissue was degraded to particle sizes of 30–40 μm while the application of 0.6% enzyme resulted in a degradation to 10–20 μm (Fig. 7).

In accordance with earlier investigations, the characteristic particle size range for squash is at 10–30 μm. In the study of the effect of time upon the distribution of squash particles according to size, the frequency peak was found to be in the range of 70–80 μm for all the three incubation periods. Since in the sample incubated for 1 h, particles of 50–60 μm dominated while after 3 h those of 70–80 mμ, of these two periods, 1 h seems the more advantageous (Fig. 8).



Figs. 7. and 8. Granulometric data and frequency distribution of particle size for winter squash treated with different endo-PG concentrations for 3 h or with 0.5% enzyme for different incubation periods (two repetitions with variety *Nagydobosi*)

2.5. Study of celery

When studying celery variety *Imperator*, the most frequent particle sizes were found in the range of 60–70 μm for the 3 endo-PG concentrations (0.06, 0.12 and 0.6%) applied (Fig. 9).

The maximum of the frequency distribution (34%) was found at 60 μm in samples treated with 0.12% enzyme. With lower or higher concentrations the maxima of frequency distribution were found to be 25–26%. In particle size ranges below 50–60 μm, the frequency of particles was nearly identical (5–7%) and this is probably due to some inhibitory effects (Fig. 9).

In the study of the effect of time, the particle size of 40 μm was found to be the most frequent after 1 and 2 h of incubation, while after 3 h, sizes between 50 and 60 μm occurred most frequently. After 1 and 2 h incubation 51 and 55%, respectively, of the particles were found in the 10–40 μm range, thus substantial difference was not found between these two incubation periods (Fig. 10).

2.6. Study of green paprika

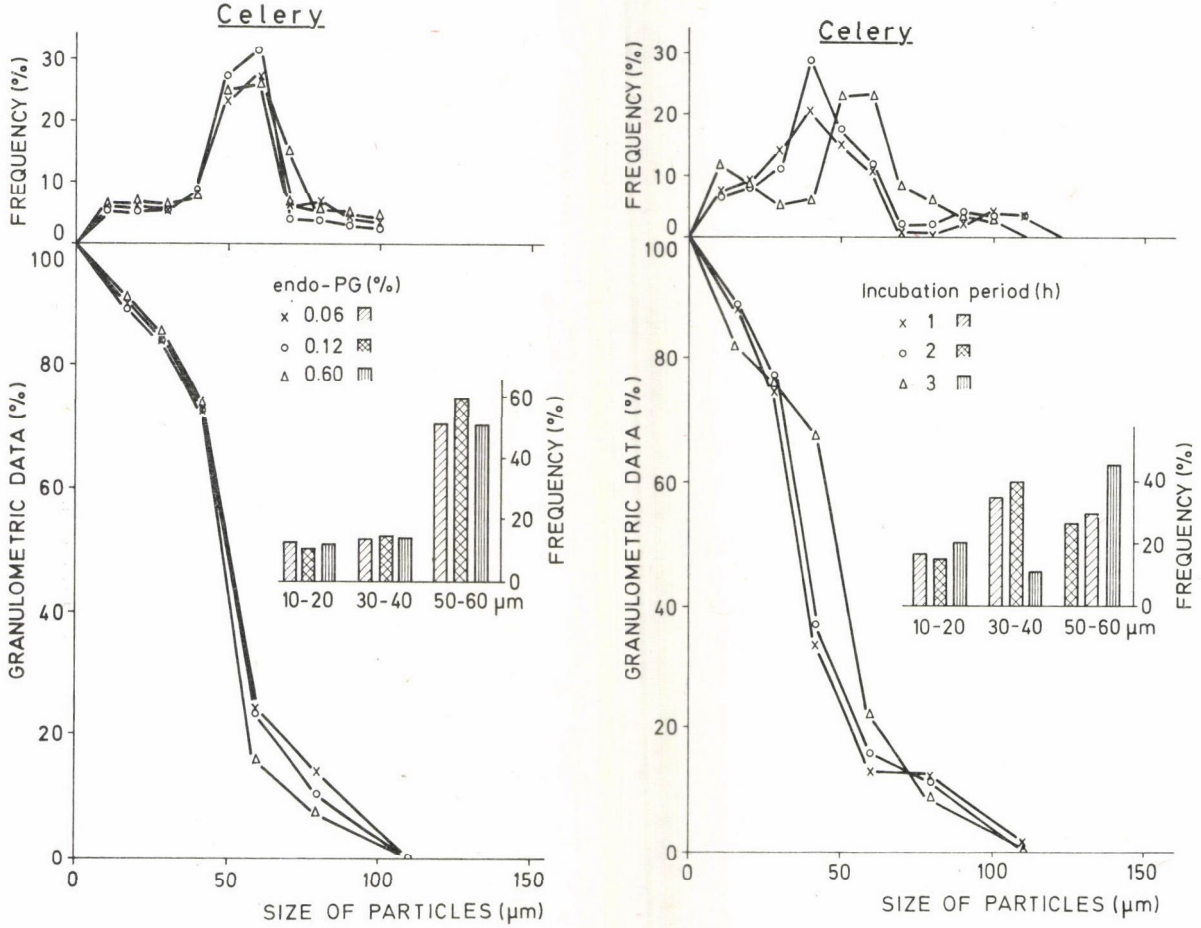
Variety *Keszthelyi fehér* was treated with 0.06, 0.12 and 0.6% enzyme preparation. Dispersoid analysis of the suspensions did not detect differences between the effects of the three concentrations. The frequency maxima for all three concentrations were found to be at 10 μm. The amount of particles in the range of 10–20 μm was about double that in the range of 30–40 or 50–60 μm. A particle of 10 μm corresponds to the characteristic cell diameter of paprika (Fig. 11).

To study the effect of incubation period, samples of the same paprika variety were incubated for 1, 2 and 3 h (Fig. 12).

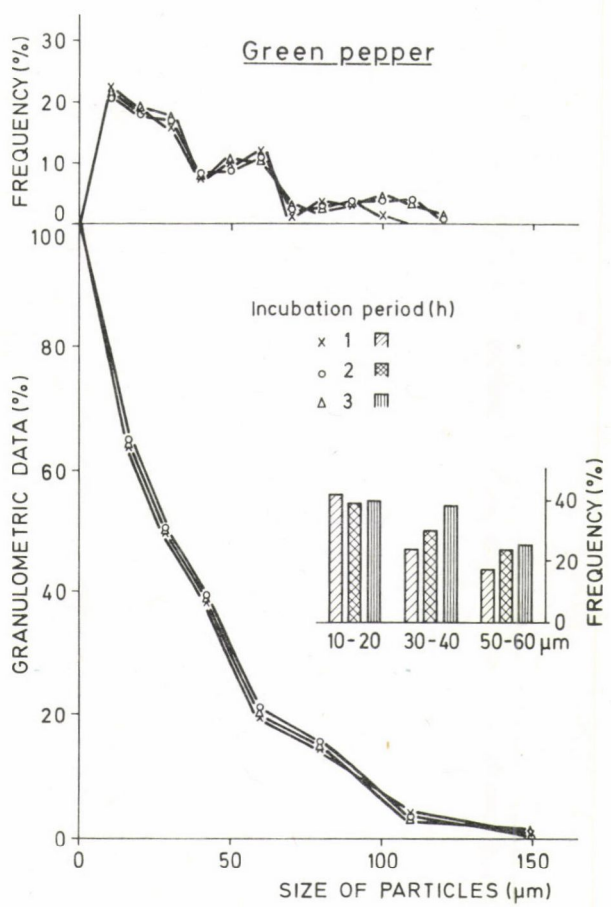
As with different enzyme concentrations, the frequency peaks were found at 10 and 60 μm particle sizes for every incubation period applied. The difference between particle sizes upon treatment at different incubation periods was not significant.

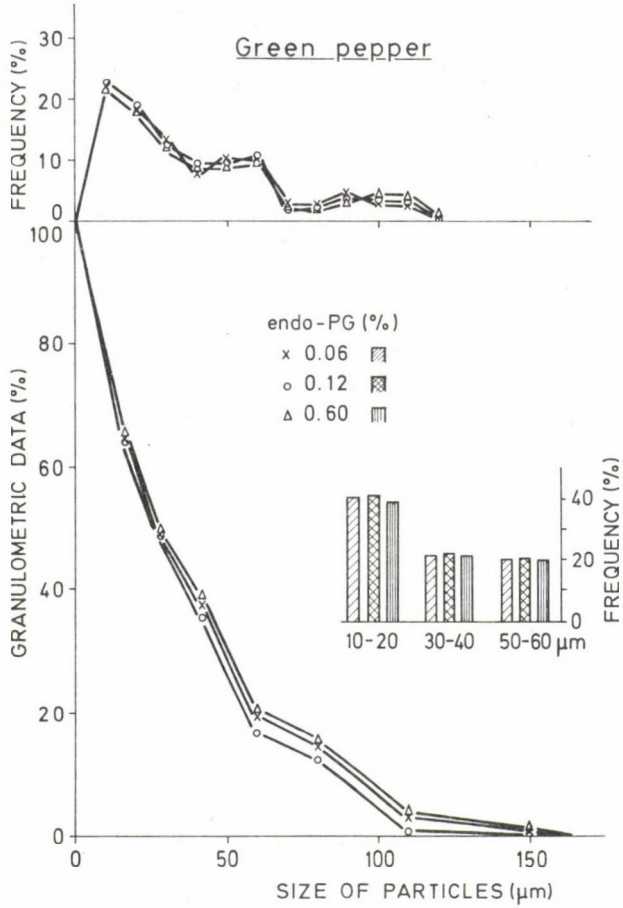
3. Conclusions

The present and earlier sedimentation tests prove the reproducibility of the method with satisfactory accuracy. The standard deviation of the granulometric data studied in seven size ranges was below 2% (Tables 1 and 2).



Figs. 9. and 10. Granulometric data and frequency distribution of particle size for celery treated with different endo-PG concentrations for 3 h or with 0.5% enzyme for different incubation periods (two repetitions with variety *Imperator*)





Figs. 11. and 12. Granulometric data and frequency distribution of particle size for green paprika treated with different endo-PG concentrations for 3 h or with 0.5% enzyme for different incubation periods (two repetitions with variety *Szentesi fehér*)

The standard deviation of average curves plotted from measurements carried out at different times with the same vegetable variety varied between 0.5 and 5% (Table 3).

In the case of carrots, the standard deviation of samples treated with 0.6% enzyme varied between 7 and 15%, when the granulometric curves represented the average values of the three varieties treated. At lower enzyme concentrations, even around 20% differences occurred between the standard deviations of curves.

Table 1

Standard deviation of the fractions of the 3 replicates during sedimentation test of the six vegetables studied disintegrated by various concentrations of endo-PG *enzyme*

Vegetables tested	endo-PG conc. (%)	Diameter of vegetable particles (μm)							Average of standard deviation (%)
		16	28	42	60	80	110	150	
		Standard deviation of the quantity of vegetable particles (%)							
Potato	0.06	0.172	2.085	0.574	1.059	1.540	3.141	1.980	1.520
	0.12	0.177	0.364	0.420	2.046	0.358	2.399	1.451	1.030
	0.60	0.210	0.196	0.098	0.684	1.165	1.100	2.023	0.782
	1.00	0.606	0.227	0.832	1.800	2.116	1.265	2.180	1.289
Green pepper	0.12	0.960	0.960	1.015	0.570	0.415	0.111	0.100	0.590
	0.60	0.375	0.715	0.529	0.570	1.640	0.020	0.115	0.566
	1.00	0.414	0.524	0.441	0.389	0.721	0.560	0.216	0.414
Squash	0.06	0.564	0.658	0.825	0.495	0.303	0.556	0.169	0.496
	0.12	0.332	0.655	0.575	0.403	0.188	0.442	0.151	0.439
	0.60	0.130	0.182	0.811	0.356	0.497	0.456	0.205	0.377
Celery	0.06	0.179	0.192	0.397	0.401	0.892	–	–	0.410
	0.12	0.141	0.187	0.374	1.300	1.787	–	–	0.757
	0.60	0.619	0.221	0.751	1.865	1.835	0.088	–	0.896
	1.00	0.605	0.613	0.913	0.684	0.640	–	–	0.691
Kohlrabi	0.06	0.214	0.485	0.936	0.167	0.335	0.636	0.128	0.411
	0.12	0.741	1.144	2.247	0.769	0.657	1.040	0.281	0.983
	0.60	0.152	1.930	0.359	1.256	1.010	1.278	0.188	0.881
	1.00	0.468	0.892	1.546	1.120	0.985	0.317	0.171	0.785
Carrots	0.06	0.150	0.463	0.899	0.466	0.725	0.380	0.112	0.460
	0.12	0.505	1.437	3.411	4.485	0.205	–	–	2.008
	0.60	0.600	3.746	7.099	8.080	0.376	–	–	3.980
	1.00	0.143	0.748	2.733	4.184	0.832	–	–	1.728

Table 2

Standard deviation of the fractions of the 3 replicates during sedimentation test of the six vegetables studied disintegrated by 0.5% endo-PG for different incubation periods

Vegetables tested	Time of incubation (h)	Diameter of vegetable particles (μm)							Average of standard deviation (%)
		16	28	42	60	80	110	150	
		Standard deviation of the quantity of vegetable particles (%)							
Potato	1	0.237	0.349	0.744	1.265	0.303	1.345	1.308	0.793
	2	0.343	1.715	0.596	1.179	2.795	2.707	1.265	1.514
	3	0.461	0.395	0.600	0.812	1.227	1.444	0.105	0.721
Green pepper	1	2.245	3.845	3.617	1.983	0.881	1.096	0.158	1.975
	2	0.620	0.507	0.632	0.319	0.456	0.882	0.122	0.506
	3	0.413	0.184	0.300	0.560	0.259	0.521	0.071	0.330
Squash	1	0.228	0.370	0.295	4.437	0.372	–	–	1.140
	2	0.434	1.253	0.361	0.341	0.982	–	–	0.674
	3	0.303	0.341	3.398	4.234	1.026	–	–	1.860
Celery	1	0.000	0.934	0.806	0.385	0.352	0.559	–	0.506
	2	0.615	0.844	0.297	0.580	0.873	0.126	–	0.556
	3	2.373	0.609	0.665	0.653	0.677	0.000	–	0.829
Kohlrabi	1	0.406	0.464	0.419	0.268	0.567	0.475	–	0.433
	2	0.477	0.152	0.729	0.381	0.290	0.427	–	0.409
	3	0.152	1.050	1.069	0.138	0.480	0.553	0.275	0.531
Carrots	1	0.251	1.362	0.226	0.241	0.182	0.532	0.359	0.450
	2	0.003	0.595	1.007	0.207	0.618	0.434	0.205	0.438
	3	0.441	0.459	0.581	0.122	0.810	0.389	0.197	0.429

Under identical treatments (incubation period: 3 h; enzyme concentration: 0.6%), the reproducibility with kohlrabi was satisfactory (in the range of 10–20 μm, the frequency of particle sizes amounted to 21.5 and 26.5%, while in the range of 30–40 μm to 38.5 and 35.5%).

Although the same variety was supposed to be used, substantial differences were observed in the frequency maxima of particle size distribution in squash. On testing the effect of enzyme concentration, the peak was found at 30 μm. This corresponded to the results of earlier investigations (ZETELAKI-HORVÁTH & URBÁNYI, 1968). When the effect of time of incubation was investigated, the granulometric curve of squash resembled that of the potato, probably due to the effect of starch with a frequency peak at 70–80 μm.

In the case of paprika, the results of the sedimentation tests belonging to two enzyme treatments carried out at two different times showed a good agreement. The slope of granulometric curves was similar and the frequency

Table 3

Standard deviation of the vegetable particles disintegrated for 3 h with various endo-PG *concentrations in two different runs, using the same variety.*

Data are the average standard deviations of the results of six sedimentation columns. In the case of carrots at endo-PG concentrations of 0.06 and 0.12%, the two different runs were made with two different varieties (average of the results of six columns), while in the case of 0.6% endo-PG, the average standard deviations represent data of three different runs using three different varieties (averages of nine sedimentation columns)

Vegetables tested	endo-PG conc. (%)	Diameter of vegetable particles (μm)				
		16	28	42	60	80
Potato variety: *Rózsa*	0.06	1.248	1.445	1.922	2.557	2.293
	0.12	0.222	0.417	1.678	4.551	4.474
	0.60	0.515	0.888	2.693	5.189	5.433
Kohlrabi variety: *Szentesi kék*	0.06	1.640	12.813	12.587	9.973	3.223
	0.12	1.859	2.270	7.201	1.245	0.445
	0.60	3.544	5.086	2.964	3.040	1.366
Squash variety: *Nagydobosi*	0.06	2.607	18.328	24.005	16.540	20.279
	0.12	4.635	20.729	21.360	14.550	18.708
	0.60	5.320	8.914	11.149	15.070	15.595
Carrot varieties: *Chantenay* *Fertődi vörös*	0.06	12.656	15.943	20.151	2.066	1.416
	0.12	14.476	12.714	12.576	12.457	7.463
Chantenay *Fertődi vörös* *Vörös óriás*	0.60	10.526	12.260	12.809	7.075	7.554

maxima of particle sizes (39, 40 and 20.5, 19.5%, resp.) were found at identical sizes (10 and 60 μm).

The results of the experiments permit the following conclusions.

The optimum enzyme concentration for particle size reduction in the majority of the vegetables tested, was found to be 0.6%. This result is in good agreement with those of earlier investigations (ZETELAKI-HORVÁTH & GÁTAI, 1977b; ZETELAKI-HORVÁTH & MOLNÁR-BIBOR, 1979) where the application of 0.5% endo-PG resulted in satisfactory breakdown and yield of solids content. With green paprika the application of 0.12% enzyme proved to be sufficient, and perhaps for celery too, the enzyme concentration may be reduced below 0.6%.

Since the use of a raw material of mixed variety may be expected in industrial processing, sedimentation analysis of the three carrot varieties were treated jointly, too.

It was found that the frequency of particle sizes between 10 and 60 μm varied between 10 and 13%. Thus it seems that, in the case of a mixed raw material, an endo-PG concentration of 0.6% is sufficient to obtain good results (Fig. 13).

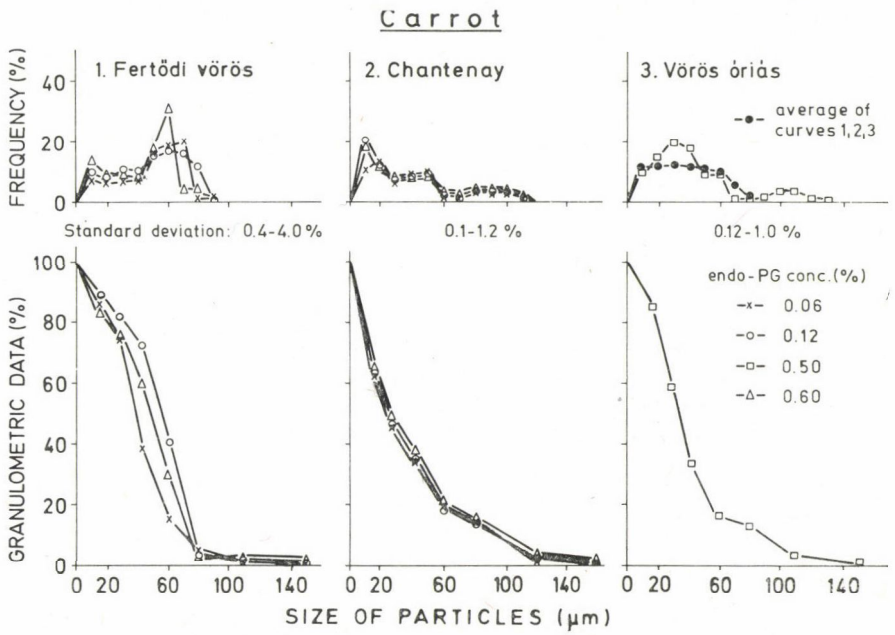


Fig. 13. Granulometric data and frequency distribution of particle size in treatment for 3 h with different endo-PG concentrations or different carrot varieties. Average frequency of particle sizes for the three varieties after treatment for 3 h with 0.6 or 0.5% enzyme

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Grateful thanks are due to lecturer Dr. György URBÁNYI for placing the sedimentation columns of the University of Horticulture, Budapest at our disposal.

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COMPARATIVE INVESTIGATIONS INTO THE ACTION OF CHYMOSIN AND A MICROBIAL MILK CLOTTING ENZYME PREPARATION ON SOME MILK PROTEINS. I. DECOMPOSITION OF α_s-CASEIN

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The degradation, by chymosin (CR) and a purified microbial coagulant from *Endothia parasitica* (MR), of α_s-caseins prepared from fresh (I), pasteurized (65 °C, 30 min) (II) and hydrogen peroxide-treated (III) skim milk was studied by polyacrylamide gel electrophoresis (PAGE) and measurement of non-protein nitrogen (NPN) content.

The release of NPN was found to depend on the method of purification of α_s-casein: if purified under mild conditions, no NPN was formed from this protein up to 60 min by either enzyme. If a certain denaturation occurred during purification, release of NPN was noticed with both enzymes, the values being higher for MR on all three substrates. For CR, the amount of NPN formed during 60 min decreased in the order III > II > I, for MR the order was III > I > II.

The electrophoretic patterns of the protein fractions formed upon the action of the enzymes were consistent with the above results. However, the differences were not only of quantitative, but also of qualitative character. The differences in the protein patterns were due partly to the higher proteolytic activity of MR and partly to changes in the properties of the substrates caused by milk treatment.

Heat treatment of milk increased the susceptibility of α_s-casein to the proteolytic action of CR, while it had an opposite effect on MR. Hydrogen peroxide treatment of milk caused initial inhibition of α_s-casein degradation by MR, but gave rise to a marked activation of the process in the later phases of the reaction which lead, in 24 h, to complete decomposition of the proteins to low molecular weight products.

The results indicate that the difference in the proteolytic action of the two enzymes can be reduced by heat treatment of the milk.

In view of the steadily increasing price of calf rennet on the world market a suitable substitute has been developed some years ago in the authors' laboratory by a submerged fermentation process using the mould strain *Endothia parasitica* (VÁMOS-VIGYÁZÓ *et al.*, 1978b). The applicability of the microbial enzyme preparation was confirmed by a series of pilot plant and large scale cheese-making experiments some results of which have been reported in this journal (KISS *et al.*, 1975; VÁMOS-VIGYÁZÓ *et al.*, 1975). In these experiments the action of the microbial preparation was found to be somewhat different, in several respects, from that of calf rennet. *E.g.*, hydrogen peroxide treated milk was coagulated at a lower rate by the former clotting agent. In order to establish optimum conditions for the utilization of the microbial coagulant, a more detailed study of its mode of action seemed expedient.

This was achieved by comparative model experiments into the decomposition of caseins prepared from fresh, pasteurized and hydrogen peroxide treated skim milk as brought about by chymosin and the purified microbial enzyme. Parts of this work dealing with changes in the electrophoretic behaviour of whole and isolated $\varkappa$-casein as well as with the accumulation of non-protein nitrogen (NPN) during the enzyme reaction had been published earlier (VÁMOS-VIGYÁZÓ *et al.*, 1979b, 1980a, b). As a result of these studies it was demonstrated that the differences in the behaviour of the two enzymes were partly due to the higher non-specific proteolytic activity of the microbial coagulant. Thus a thorough study into non-specific proteolysis, the tertiary phase of the milk clotting process, seemed indicated using isolated α_s- and β-caseins as substrates of the reaction, since these proteins are not affected in their chemical composition by the previous phases of the clotting process. The present paper reports on the results obtained with α_s-casein, using the same techniques as with whole and $\varkappa$-casein.

1. Materials and methods

1.1. Milk

Commercial milk supplied by a Budapest dairy plant was used in the fresh state (I), after pasteurization at 65 °C for 30 min (II) and after hydrogen peroxide–catalase treatment (III) (VÁMOS-VIGYÁZÓ *et al.*, 1978a), resp., for the preparation of acid casein and therefrom α_s-casein.

1.2. α_s-casein

Two methods were applied to prepare α_s-casein. The urea fractionation method of ZITTLE and co-workers (1959) followed by the purification procedure of ZITTLE and CUSTER (1963) yielded a preparation contaminated probably by β_s-casein as can be seen also from the electrophoretograms published by above authors. Therefore, a simple purification method based on the selective insolubility of α_s-casein in $CaCl_2$ solutions at temperatures between 0 and 4 °C was developed in this laboratory by which an electrophoretically and ultracentrifugally homogeneous protein could be obtained. Details of the method have been described elsewhere (VÁMOS-VIGYÁZÓ *et al.*, 1979a).

1.3. The enzyme preparations

The crystalline chymosin preparation applied throughout the experiments was purchased from SIGMA (USA). The microbial coagulant was obtained

from a crude enzyme preparation manufactured at the fermentation plant of the factory PHYLAXIA, Budapest, by gel filtration on *Sephadex* G 10 and G 100, repeated ion exchange on DEAE-cellulose and finally, desalting on *Sephadex* G 10 (KISS *et al.*, 1976). The specific activities as related to protein content were 830 000 SU g^{-1} for chymosin and 240 000 SU g^{-1} for the liquid preparation of microbial origin (SU = *Soxhlet* unit; 1 SU = the amount of milk coagulated by 1 g or 1 cm^3 of enzyme preparation in 40 min at 35 °C).

1.4. Enzymatic decomposition

For electrophoretic studies the reaction mixtures contained 4 mg cm^{-3} α_s-casein. The amount of enzyme added per cm^3 of the reaction mixture was sufficient to coagulate 0.6 cm^3 of fresh skim milk in 30 min at 35 °C. For NPN-determinations, the concentrations of both substrate and enzyme were about 5-fold. The reaction was carried out at pH 6.0 and 35 °C.

Samples for the electrophoretic runs were taken after 30, 60, 120, 180 and 1440 min and for NPN-determinations after 5, 10, 15, 30 and 60 min. "0"-min samples were prepared under identical conditions by the addition of heat inactivated enzyme to the substrate solution.

The samples taken at intervals for electrophoresis were diluted in a volume ratio of (1 : 1) with pH 8.6 Na-veronal – hydrochloric acid buffer (ionic strength 0.005) to inactivate the enzyme, while those withdrawn for NPN-determination were mixed in the same ratio with 24% trichloro-acetic acid (TCA).

The enzyme reactions were carried out in triplicate.

1.5. Polyacrylamide gel electrophoresis (PAGE)

To aliquots of 0.2 cm^3 of the samples (0.5 mg of protein) 0.1 cm^3 of 2-mercapto ethanol were added 15 min before starting the electrophoretic runs. The samples were applied on top of the gels polymerized in the tubes of 16 cm length of the apparatus ACRYLOPHOR (PLEUGER, Belgium), after mixing with 45% glycerol and the marker dye bromophenol blue.

Both the gel and the buffer mentioned above were "continuous". The gels contained 5% acrylamide, 0.13 bis-acrylamide and were 5 molar with respect to urea. The runs took 4 to 5 min at a current intensity of 5 mA per gel tube and 15 °C. The sample end of the gels was at the cathode side. Staining was performed with Amido black, destaining of the background with a 7% acetic acid solution. Details of the procedure had been given earlier (VÁMOS-VIGYÁZÓ *et al.*, 1978a).

1.6. Determination of non-protein nitrogen (NPN)

The nitrogen content of the reaction products soluble in 12% TCA was determined in the filtrates by the *Kjeldahl* method and expressed as % of total nitrogen content.

2. Results

2.1. Accumulation of NPN during the decomposition of the α_s-casein substrates by the two enzymes

The values of NPN released during the enzymatic decomposition of α_s-caseins prepared from skim milk treated in different ways according to ZITTLE and co-workers (1959) and ZITTLE and CUSTER (1963) are shown in Fig. 1.

During identical periods in all the cases considerably less NPN was set free by chymosin than by the microbial rennet. NPN formation by chymosin was slightest with α_s-casein obtained from fresh milk (I) and strongest with that from hydrogen peroxide treated milk (III). The largest amount of NPN produced in 60 min by the microbial enzyme was equally observed with the substrate prepared from hydrogen peroxide treated milk (III), while the lowest one was obtained with α_s-casein from pasteurized milk (II). However, during

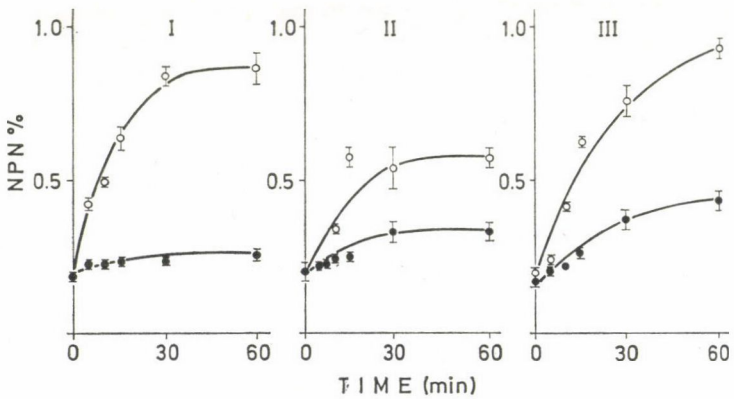

Fig. 1. Release of non-protein nitrogen (NPN) from α_s-caseins prepared by urea fractionation (ZITTLE *et al.*, 1959; ZITTLE and CUSTER, 1963) from fresh (I), pasteurized (II) and hydrogen peroxide-treated (III) skim milk upon the action of chymosin (full circles) and the purified microbial coagulant (open circle). *Experimental conditions:* 20 mg cm^{-3} α_s-casein; enzyme per cm^3 sufficient to coagulate 3 cm^3 of fresh skim milk in 30 min at 35 °C. Temperature of the reaction: 35 °C, pH: 6.0. The "0" time sample contained heat-inactivated enzyme. NPN was determined by the *Kjeldahl* method and expressed as % of total N. The reaction was carried out in triplicate. The vertical bars represent standard deviations

the initial phase of the reaction with this enzyme, NPN was formed at a higher rate from substrate I than from substrate III. The release of NPN from substrates I and II continued with both enzymes for 15 and 30 min, resp., while with substrate III the values increased till the end of the observation period (60 min).

The α_s-caseins prepared according to the authors' method practically did not give rise to NPN formation during 60 min of incubation with either enzyme.

2.2. Investigations by PAGE into the enzymatic degradation products of α_s-caseins obtained from milks treated in different ways

The degradation products detectable by protein staining as formed upon the actions of the two enzymes were studied only with the substrates prepared according to the authors' method.

The changes in the electrophoretic pattern of α_s-casein I from fresh milk as occurred upon the action of chymosin are shown in Fig. 2.

The electrophoretically homogeneous protein was split by the enzyme into several fractions mainly of lower relative mobilities than that of the original substrate. Only one fraction was of higher mobility.

The degradation of the same substrate by the microbial enzyme preparation is shown in Fig. 3.

It can be clearly seen that this enzyme brings about a more pronounced decomposition yielding a multitude of fractions of mobilities lower and higher than that of the original protein. The zone corresponding to the initial compound is getting visibly fainter and thinner to be practically entirely transformed by the end of the observation period (1 440 min).

The degradation of α_s-casein II as caused by chymosin is represented in Fig. 4.

The relative mobility of this substrate is lower than that of α_s-casein I. During the reaction it is split into a maximum of 9–10 fractions, partly of lower and partly of higher mobilities than that of the initial compound. Part of the original substrate is still detectable after 1 440 min of incubation with the enzyme.

The changes occurring in the electrophoretic behaviour of the same substrate upon the action of the microbial enzyme are shown in Fig. 5.

The pattern was roughly similar to that obtained for this substrate with chymosin. The fractions formed upon the action of the microbial enzyme showed partly a more positive and partly a more negative net electric charge as compared to the initial protein. Their maximum number was 10–11. A small part of the initial protein was still detectable after 1 440 min.

The decomposition products of α_s-casein III from hydrogen peroxide treated milk as formed upon the action of chymosin can be seen in Fig. 6.

Although prepared in exactly the same way as α_s-caseins I and II, α_s-casein III gave several fractions in the electric field, the main component being accompanied by a number of proteins of lower relative mobilities. The relative mobility of the main component was identical with that of α_s-casein I. A large part of the principal fraction persisted up to 180 min, however, after 1440 min only traces of this compound were discernible along with some fractions of lower relative mobilities.

The time course of the degradation of α_s-casein III by the microbial enzyme is followed in Fig. 7.

During the initial period of the reaction the decomposition of the main component seems to be somewhat retarded as compared to the sample incubated with chymosin. In this case fractions more negatively charged than the principal component were also formed to a considerable extent. Both the remainders of the main component and the degradation products were detectable after 180 min, however, no band staining with Amido black could be found after 1 440 min. (The respective test tube containing the transparent gel only is not shown in the Figure.)

3. Conclusions

The data obtained with respect to the formation of NPN show a strong dependence of the enzymatic degradability of α_s-casein on the method of its preparation. This might be one of the causes of the discrepancies found among the data published in the literature on the amounts of NPN released from α_s-casein by the action of different milk clotting enzymes. Differences in experimental conditions (pH, concentrations and ratios of substrate and enzyme, purity of the latter) as applied by the different authors might equally bear on the results.

Thus, *e.g.*, according to VANDERPOORTEN and WECKX (1972) the initial NPN-content of about 0.8% of the reaction mixture containing α_s-casein as substrate was increased in 120 min to more than 2% and nearly 7%, by chymosin and a milk clotting enzyme preparation from *Endothia parasitica*, resp. According to MICKELSEN and FISH (1970) as well as PAQUET and ALAIS (1978), out of several milk clotting enzyme preparations of microbial origin, the one obtained from *Endothia parasitica* liberated the highest amount of NPN from α_s-casein, whereas no NPN was formed at all from this compound upon the action of chymosin. EL-NEGOUMY (1968) and ITOH (1972) reported on similar results with respect to chymosin, while LEDFORD and co-workers (1968) found with this enzyme, at pH 6.0, a considerable increase in the amount of the products of proteolysis soluble in 5% TCA. All these data were obtained with α_s-casein prepared from fresh milk.

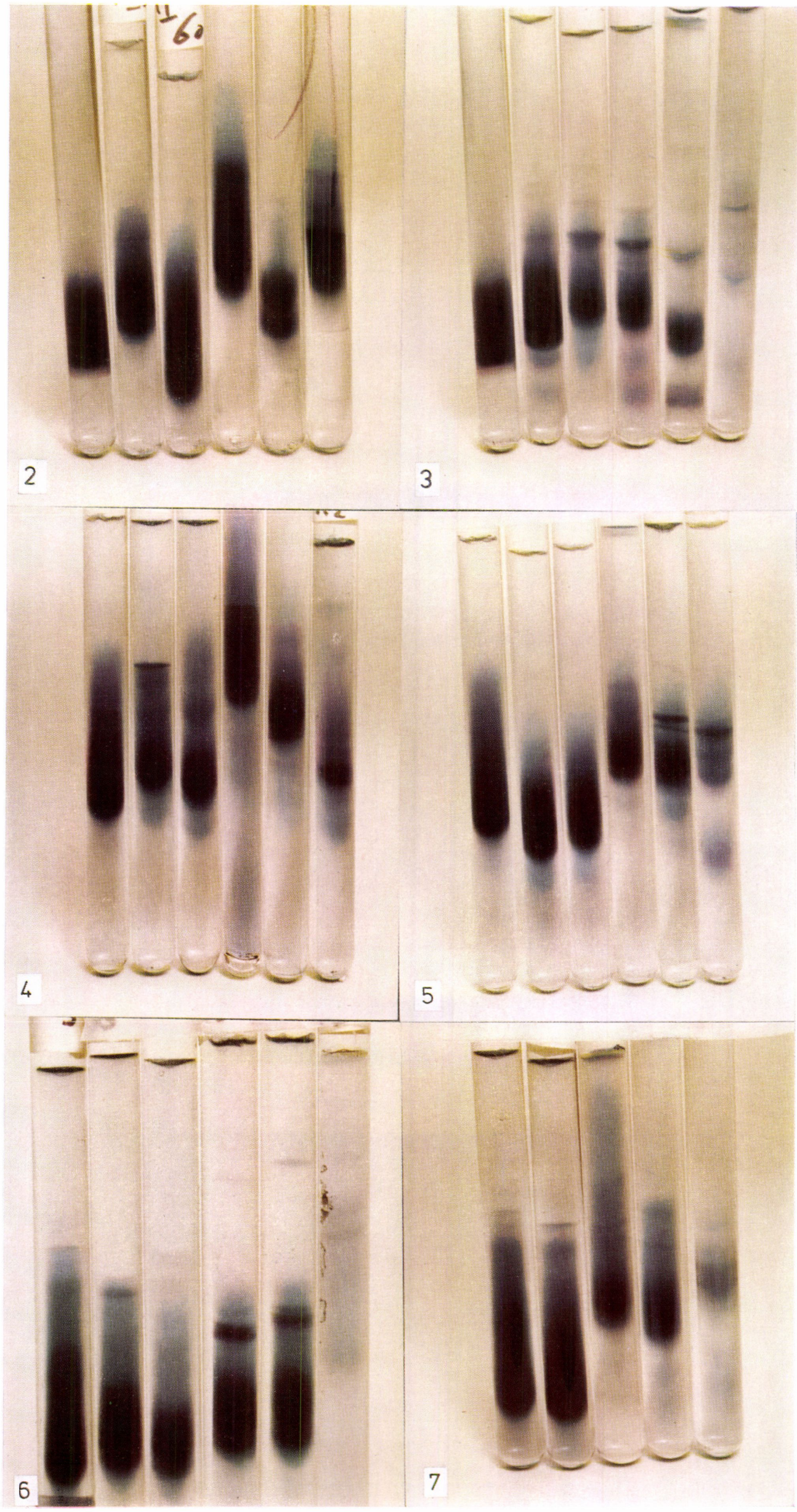

Fig. 2. Changes induced by chymosin in the electrophoretic behaviour of α_s-casein prepared from fresh skim milk by selective precipitation with $CaCl_2$ (VÁMOS-VIGYÁZÓ *et al.*, 1979a). *Experimental conditions*: 4 mg cm^{-3} α_s-casein; enzyme per cm^3 sufficient to coagulate 0.6 cm^3 of fresh skim milk in 30 min at 35 °C. For the rest of reaction conditions see legend to Fig. 1. Electrophoresis: 5% polyacrylamide, 5 *M* urea, pH 8.6. Samples diluted prior to application with 45% glycerol and 2-mercapto ethanol (1 : 1 : 1). Sample size: 0.5 mg protein per gel. Run: 45 min, 5 mA/gel, 15 °C. Staining: Amido black. The tubes show, from left to right, the patterns of samples taken after „0", 30, 60, 120 and 1 440 min of incubation

Fig. 3. Changes induced by the microbial coagulant in the electrophoretic behaviour of α_s-caseins prepared from fresh skim milk. For experimental conditions and explanations see Fig. 2

Fig. 4. Changes induced by chymosin in the electrophoretic behaviour of α_s-casein prepared from pasteurized skim milk. Pasteurization: 65 °C for 30 min. For the rest of experimental conditions and for explanations see Fig. 2

Fig. 5. Changes induced by the microbial coagulant in the electrophoretic behaviour of α_s-casein prepared from pasteurized skim milk. For experimental conditions and explanations see Figs. 2 and 4

Fig. 6. Changes induced by chymosin in the electrophoretic behaviour of α_s-casein prepared from hydrogen peroxide-treated skim milk. Hydrogen peroxide treatment according to VÁMOS-VIGYÁZÓ and co-workers (1979a). For the rest of experimental conditions see Fig. 2

Fig. 7. Changes induced by the microbial coagulant in the electrophoretic behaviour of α_s-casein prepared from hydrogen peroxide-treated skim milk. For experimental conditions and explanations see Figs. 2 and 6

The results obtained for the NPN released from α_s-casein prepared according to ZITTLE and co-workers (1959) as well as ZITTLE and CUSTER (1963) are in agreement with earlier observations on whole casein (VÁMOS-VIGYÁZÓ *et al.*, 1980a). In the paper cited it was shown that the non-specific proteolytic activity of the microbial enzyme was considerably higher than that of chymosin, further, that this activity of the former enzyme apparently diminished on substrates prepared from pasteurized milk. Thus pasteurization seemed to reduce the differences in the actions of the two enzymes. Finally, it was found that hydrogen peroxide treatment of the milk enhanced – after initial inhibition – the proteolytic activity of the *Endothia parasitica* enzyme towards some of the casein components.

On the other hand, the results presented demonstrate that proteolysis of α_s-casein as caused by the microbial coagulant can be prevented by mild conditions of purification of this protein.

The electrophoretic investigations equally showed, in agreement with the findings of others (MICKELSEN & FISH, 1970), differences between the actions of the two enzymes. These were, however, mainly of quantitative character – contrary to the results obtained with whole and $\varkappa$-casein for the primary phase of the reaction (VÁMOS-VIGYÁZÓ *et al.*, 1980a, 1980b). Some authors (VANDERPOORTEN & WECKX, 1972; PAQUET & ALAIS, 1978) detected, in agreement with this study, decomposition products of higher as well as of lower mobilities than that of α_s-casein, while others (MICKELSEN & FISH, 1970) mentioned only the formation of compounds of higher mobilities. These contradictions might also be accounted for by different reaction conditions, by the different degrees of purity of the enzyme preparations or, perhaps, by differences in the electrophoretic systems applied.

No data were available on the role milk pretreatment might play in the enzymatic degradability of α_s-casein. According to the data presented this role is less important for this protein than for whole or β-casein.

Summarizing the results it can be said that the action, on α_s-casein, of the milk clotting enzyme from *Endothia parasitica* as developed in this laboratory differs from that of calf rennet owing to the higher non specific proteolytic activity of the former. However, essential differences can be noticed only, even when applying multiples of the enzyme and substrate concentrations used in cheesemaking, if the protein had been considerably denatured during purification. Thus, as proven by a great number of cheesemaking trials, such differences have not to be taken into account in practice. It was further demonstrated, as to our knowledge for the first time, that the degradability of α_s-casein by milk coagulants was strongly dependent on the mode of preparation of this protein, *i.e.* on its denaturation during purification.

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BOOK REVIEWS

Recommendation for chilled storage of perishable produce

International Institute of Refrigeration (177, boulevard Malesherbes – 75017 Paris, 1979: 148 pp)

This is a completely recast edition of the well-known brochures entitled "Recommended conditions for cold storage of perishable produce" (1959 and 1967). It deals with the chilled storage of fruits and vegetables, of meat, poultry and egg, of fishery products, of dairy products, of cut flowers, of seeds and of some miscellaneous items. The booklet contains a wealth of data in a most concise form, representing present knowledge of optimal storage conditions of the above groups of products.

Before going into detail, the brochure gives a most useful outline of the various terms used and of the factors influencing the result of cold storage. The general introduction deals specifically with the expected or practical storage life and the effect of initial quality on storage life. The respective roles of the cooling-down period, of storage temperature, of relative humidity, air circulation, ventilation, packaging and stacking are discussed. The effects of volatile substances as well as that of storage hygiene are specifically mentioned. Conditions leading to condensation on removal from cold storage are also described.

The booklet contains the work of some thirty experts of international renown. Dr. J. A. MUNOZ-DELGADO (Spain) served as the General Coordinator of the task force, while Drs. R. ULRICH (France), L. BØGH-SØRENSEN, M. JUL (Denmark), I. J. CONNELL (United Kingdom), G. GROSCLAUDE (France), A. PAULIN (France), and D. COME (France) acted as coordinators for the various chapters.

Dr. J. ESPINOSA (Spain) fulfilled the role of the Secretary of the Working Group responsible for the collaborative venture.

The book is a bilingual treatise, both text and tables can be read in English and French.

This publication supplies a great want and should be on the bookshelf of all those working in areas connected with chilled storage of foods and other perishable products.

K. VAS

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